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Characterization of the hypoxically inducible alanine aminotransferase and its role in flood tolerance in plants

by

© **Douglas Gordon Muench**

A thesis sumbitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Genetics

Edmonton, Alberta

Spring 1994

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To Michele and Connor

Abstract

The hypoxically inducible barley alanine aminotransferase (AlaAT-2; EC 2.6.1.2) was purified to homogeneity from barley root tissue by ammonium sulphate precipitation and a series of chromatographic columns. The purified protein had a specific activity of 2231 IU/mg protein, and was very specific in the substrates it used. AlaAT-2 was shown to be a homodimer composed of 50 kD subunits. A full-length barley AlaAT-2 cDNA clone was isolated, and had an open reading frame of 1446 base pairs, giving a predicted protein molecular mass of 52,885 Da. The coding region of the cDNA was 88% identical at the nucleic acid level and 90% identical at the amino acid level to that of the light inducible AlaAT cDNA from *Panicum miliaceum* leaves. A maize AlaAT genomic DNA clone was isolated and a partial sequence analysis of the coding region showed 95.1% and 87.9% identity to the the *P. miliaceum* and barley cDNAs, respectively.

The 6-fold increase in AlaAT activity after 96 h of hypoxia was similar to that of AlaAT-2 protein concentration, as indicated by protein gel immunoblots. AlaAT-2 mRNA concentration increased almost 4-fold during 24 h of hypoxic stress, followed by a gradual decrease in mRNA levels in the following 72 h. This pattern of AlaAT-2 protein and mRNA production indicated that the AlaAT-2 protein is either very stable in hypoxic cells, or there is an increase in translation rate upon prolonged hypoxia. The hypoxically inducible AlaAT-2 in the C_4 plant *P. miliaceum* had the same electrophoretic mobility as the light inducible isoform. AlaAT-2 is the only hypoxically

inducible protein known to be inducible by light as well.

In barley and wild rice, hypoxia resulted in the accumulation of substantial amounts of ethanol. Alanine also accumulated in tissues of both of these species. Malate concentrations increased in wild rice roots and leaves, however it did not accumulate over the long-term in hypoxic barley roots. Lactate, aspartate and succinate were not significant end-products of hypoxic metabolism in either of these two species. The universal synthesis of alanine and ethanol in plants exposed to hypoxic stress suggests that these two compounds have important roles in a plant's ability to tolerate short-term flood conditions. Cytoplasmic acidosis and the availability of adequate carbohydrate reserves maybe important in long-term flood-tolerance.

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1 Introduction

In order to cope with an ever-changing environment, plants have evolved a number of strategies necessary for survival and reproduction. They possess adaptive responses to temperature, salinity, ultraviolet light and drought stress among others, and at some point during development, plants often experience reduced oxygen (hypoxic) conditions. Hypoxic stress occurs primarily during periods of flooding, where all or part of the plant is submerged, although low oxygen concentrations can be found in fine and compacted soils, or during ice cover (Armstrong 1979, Andrews and Pomeroy 1990, Jackson and Drew 1984). Under these conditions plants are unable to obtain sufficient oxygen necessary for normal respiration, and will survive only a short time unless a strategy is in place to cope with this stress (Hook and Crawford, 1978). The economic impact of low oxygen stress on crop production is high, as soil waterlogging and flood damage was responsible for 18.5% of insurance indemnities distributed in the United States over a 40-year period (1939 to 1978), second only to the yield loss caused by drought (Boyer 1982).

Plants have developed anatomical, physiological and metabolic mechanisms to survive varying degrees of hypoxic stress. Aquatic plants (eg. rice, [*Oryza sativa* L.]) can survive several days without oxygen, whereas other species (eg. pea, [*Pisum sativum* L.]), survive only hours under these conditions. The mechanisms of coping with these conditions generally involve the ability of the cell to maintain its ultrastructural integrity and energetics without oxygen (biochemical tolerance), and/or by the ability of the plant to avoid low oxygen conditions, perhaps by the transport of oxygen from oxygenated parts of the plant to those which are suffering oxygen deficiency (structural tolerance, Vartapetian et al. 1978). Evidence suggests that biochemical tolerance is required until the flood stress passes, or until the plant can undergo the structural changes necessary to reoxygenate the stressed tissue. One established structural change important for a plant's survival under hypoxic conditions involves the translocation of oxygen from aerobic parts of the plant (e.g. aerial leaves) to tissues containing low levels of oxygen (e.g. roots) through air channels (aerenchyma) located in the cortex of roots and shoots (Justin and Armstrong 1987).

There are a handful of plant species, which when completely submerged, have tissues which can survive long periods without any oxygen at all (several days up to a month). These tissues have evolved biochemical tolerance to low oxygen stress. The rice seedling coleoptile (first leaf), for example, can survive and elongate for several days without oxygen (Taylor 1942). Under these conditions, the coleoptile has the ability to synthesize proteins, lipids, saturated fatty acids and membrane structures such as mitochondrial cristae (Jackson and Pearce 1991). Even those plants which are considered "non-tolerant" to flooding can withstand at least a few hours without oxygen and, therefore, possess the biochemical mechanisms for cell viability under these conditions.

It is the differential ability of plants to survive without oxygen which is not well understood and has been the scope of much research. Historically, attention has been placed on the fermentative pathway leading to the production of ethanol. However, since ethanol production is high in both "tolerant" and "non-tolerant" species, it is thought that other mechanisms are important for longer-term viability of plant cells. As a result, many recent studies have looked at alternative pathways of hypoxic metabolism. In addition, molecular biology has contributed a substantial amount in studying the coordinate regulation of hypoxically inducible genes, and molecular biology provides significant information as to the importance of several pathways used during hypoxic stress.

To this point, I have used the term "hypoxic" rather loosely when describing the oxygen levels. Several researchers have attempted to develop consistent terminology to describe the degree of oxygen stress a plant encounters in a waterlogged environment. The terminology of Pradet and Bomsel (1978) may most adequately describe these conditions. They considered conditions as "hypoxic" when the oxygen concentration limits the production of ATP by oxidative phosphorylation in mitochondria, and "anoxic" when oxygen concentrations are so low that mitochondrial ATP production is negligible relative to that generated by glycolysis and fermentation. The term "anaerobic" describes an oxygen-free condition. According to Andrews et al. (1993), some authors have described the oxygen status of cells and tissues in their experiments as hypoxic, when in fact they are probably anaerobic, and anaerobic when they are likely hypoxic. In addition, since quantitative descriptions about the concentration of oxygen used to induce hypoxia are not often provided in publications, it is often difficult to determine the degree of oxygen stress used in their experiments.

Despite the differences in oxygen conditions used by different experimentors, each of them appears to be common in that they induce an "anaerobic response", as seen by the accumulation of certain metabolic end-products and the expression of several "anaerobic proteins". They differ, however, in the viability of the stressed tissue, as hypoxically treated tissues are known to survive longer than anaerobic ones (Hole et al. 1992, Johnson et al. 1989, Saglio et al. 1988). Because of the variable oxygen concentrations used in experiments of this type, I will use the general term "hypoxic" to describe low oxygen conditions throughout this chapter. However, in situations where the concentration of oxygen is important in demonstrating a certain point, a specific description of the actual concentration will be provided.

The review of the literature described in this section will attempt to address the significant responses of plants to flooding, including the anatomical, physiological, biochemical and molecular responses that are of relevance to this thesis. There is a large number of publications on the topic of flood tolerance, and these studies describe the hypoxic response of many species and tissue types. As a result, generalizations are made throughout this chapter when describing the response of plants to low oxygen stress.

1.1 The Hypoxic Root Environment

Well drained soils contain 10 to 60% gas by volume, and are well aerated because of rapid diffusive gas exchange between soil and air. The diffusion of air into the soil virtually ceases when the gaseous pore space is reduced as a result of waterlogging (Ponnamparuma 1984), and under these conditions, aerobic microorganisms and plant roots rapidly consume the remaining oxygen in the soil (Drew and Lynch 1980). The soil profile, however, is not completely devoid of oxygen, as a thin layer at the soil surface remains oxygenated. Generally, only the upper few millimeters of a submerged soil is oxygenated, although if the surface water contains significant oxygen concentrations (due to wave action or water flow) an oxygenated soil surface layer of up to a few centimeters can result in cool, low organic matter soils (Ponnamperuma 1984). The thickness of this zone is determined by the ratio of oxygen supply from the overlying air or water to the O_2 consumption in the soil (Ponnamperuma 1984). Oxygen consumption depends primarily on the respiration rate of aerobic bacteria, which in turn depends on the soil temperature and the availability of organic matter and soil minerals (Drew and Lynch 1980). Soils which are wet or waterlogged rather than submerged may have higher levels of oxygen, depending on the degree of water saturation.

Much of the soil profile in a newly flooded region can be devoid of oxygen in a few hours (Meek et al. 1983) to a few days (Blackwell 1983). Once the oxygen has been removed, various populations of facultative and obligate anaerobic microorganisms utilize sequencially other terminal electron acceptors to respire, and do so in the order of their redox potentials. Typically NO_3^- is first reduced to NO_2^-, followed by MnO_2 to Mn_2^+, and then $Fe(OH)_3$ to Fe_2^+. In very reduced soils, SO_4^{2-} can can be reduced to S^{2-} and released as H_2S (Drew and Lynch, 1980). Each of these and other products accumulate in large enough amounts to be potentially phytotoxic, by inhibiting respiration and nutrient uptake. Denitrification, by the conversion of NO_3^- to NO_2^-, has very dramatic effects on the health of the plant, as this process decreases the amount of nitrogen in a form available to the plant. Many other substances produced by anaerobic bacteria likely influence plant growth as well (Ponnamperuma 1984). As a result, it is apparent that when a plant is exposed to hypoxic soil conditions, it must not only deal with a lack of oxygen, but must contend with several other factors as well.

Many studies looking at the effects of hypoxia on plants, including those found in this thesis, involve the use of a hydroponic culture system to reduce the oxygen levels surrounding the plant. This treatment is known to stimulate many of the same physiological responses that are seen in naturally flooded plants (Trought and Drew 1980). However, since the deleterious factors of soil flooding mentioned in the previous paragraph are greatly reduced or eliminated in liquid culture conditions, these studies consider only a part of the changes that a plant undergoes under naturally flooded conditions (Mendelsson et al. 1981). As a result, when the data of liquid culture experiments are used in investigating a plant's natural response to low oxygen conditions, it should be recognized that some of these natural stresses are not being evaluated. Alternatively, these conditions are relatively specific in determining the response of a plant to low oxygen stress only, without the effect of other external variables.

1.2 Physiological Responses to Flooding

Rice and other aquatic species thrive naturally in flooded environments where the surface water can be several inches to several feet in deep. Rice has the unusual ability to germinate in environments completely devoid of oxygen, although germination is not normal, as only an unopened coleoptile emerges. The ability of the coleoptile to grow and maintain cell integrity without oxygen is fuelled by an efficient fermentative respiration of reserves transported from the seed (Atwell and Greenway 1987). The coleoptile continues to grow by cell elongation, as it attempts to reach the water/air interface to allow oxygen transport through aerenchyma to the rest of the plant. This elongation is due to water uptake by the cell, probably mediated by tissue accumulation of low molecular weight solutes such as alanine and putricine (Menegus et al. 1984, Menegus et al. 1993). Coleoptile elongation is important for a submerged plant to gain access to oxygen, carbon dioxide and light. The root system of rice does not elongate under anoxic conditions, and the root apex dies quickly. Lateral root meristems, however, remain viable and resume normal growth upon reoxygenation (Jackson and Drew 1984).

Shoots and roots of plants such as wheat do not tolerate hypoxic conditions, even when the leaves are in contact with air. Features symptomatic of shoot hypoxia include chlorosis (yellowing), leaf senescence, wilting, epinasty (downward growth), and a decrease in leaf extension (Jackson and Drew 1984). It is thought that some of these responses are adaptive, whereas others are a consequence of flooding damage to the roots. Since the function of the root is to supply water, nutrients and hormones to the shoot, root hypoxia is likely to be the cause of all of these morphological responses. Hypoxic plant roots have a greatly reduced ability to take up soil water (Drew and Stolzy 1991), and in response to this, leaf stomata closure, wilting and epinasty are thought to decrease the amount of water loss through the leaves. Hypoxic conditions stimulate ethylene accumulation in the roots, which is then translocated into the shoots to induce these water conserving responses (Jackson and Drew 1984). A decrease in the rate of photosynthesis is also an early response to flooding (Kozlowski 1982). Chlorosis, senescence and decreased growth are thought to result from a reduction in soil nutrients, particularly nitrogen, as Trought and Drew (1981) found that these responses could be seen in plants that were grown in aerated hydroponic culture lacking nitrate. They concluded that nitrate deficiency resulted from a decrease in nitrate assimilation in hypoxic conditions in nature, and that this deficiency was the cause of flooding injury. The limited ability of anoxic roots to absorb nutrients is believed to result from the inability of the cells to produce sufficient ATP to pump nutrients across the cell membrane (Jackson and Drew 1984). Although some ion transport can occur hypoxically, it is thought to occur passively by mass flow across the cell membrane as a result of transpirational water loss through the leaves (Trought and Drew 1981). In addition, since soil denitrification occurs in flooded environments, it is thought that this also restricts the availability of nitrate to the plant.

Reduced amounts of ATP in tissues exposed to hypoxic conditions also limits the translocation of sugars through the phloem. The availability of fermentable substrates to the hypoxic tissue is then halted, thus decreasing the rate of glycolysis. Rice coleoptiles, however, are able to transport sugars from the seed which allows for continued glycolysis. In addition, many tolerant species which possess rhizomes and tubers are known to contain substantial amounts of starch, and can utilize it to fuel glycolysis and remain viable for up to a month in hypoxia (Barclay and Crawford 1983). Hypoxia experiments studying the survival of rice coleoptiles, roots and leaves excised from the seed, showed that the viability of all of these tissues was greatly increased when 3% glucose was added to the hypoxic solution (Webb and Armstrong 1983, Vartapetian et al. 1978). These researchers speculated that under hypoxic conditions a shortage of a fermentable carbon source, rather than a lack of oxygen, is the cause of cell death.

The synthesis of several hormones in the roots is also affected by hypoxia (Reid and Bradford 1984), and this undoubtedly plays a significant role in shoot behavior. Flooding of roots results in the accumulation of abscissic acid (ABA, Zhang and Davies 1987), and ethylene (Bradford and Yang 1987) in the roots, while auxin and cytokinin decrease (Reid and Bradford 1984). ABA has been implicated in regulating gene expression under conditions of salinity (Singh et al. 1987), freezing (Mohapatra et al. 1988) and drought (Drew 1990), although its role in flood tolerance is unknown. Ethylene has a central regulatory role in underwater shoot elongation and aerenchyma formation, and is present at relatively high levels in hypoxic roots. The exact role of these hormones on the leaf hypoxic response is unknown.

1.3 Structural and Anatomical Responses to Flooding

Of the structural and anatomical adaptations to flooding stress described, aerenchyma formation is likely to be the most important factor in alleviating oxygen stress. Aerenchyma forms as gas filled spaces (lacunae) in the cortical tissues of roots, stems and leaves either by cell separation during development (schizogeny) or by cell lysis and dissolution (lysogeny), thus forming continuous columns of air within the tissue (Armstrong 1979, Fig. 1.1). Aerenchyma is found in tissues of wetland and non-wetland species, and allows the transport of atmospheric or photosynthetically derived oxygen from oxygenated tissues to oxygen-starved tissues. The transported oxygen would then restore partial or full mitochondrial function of cells, thus reestablishing normal physiological functions such as phloem transport and ion uptake (Armstrong 1971). A well developed network of aerenchyma can also bring oxygen to the soil immediately surrounding the root, thus decreasing the concentration of toxins and other deleterious compounds

This figure has been removed because of copyright restrictions. The photograph that was to be presented here is found in:

Gaynard TJ and Armstrong W (1987) Some aspects of internal plant aeration in amphibious habitats. In RMM Crawford, ed, Plant Life in Aquatic and Amphibious Habitats, Blackwell Scientific Publishers, Oxford, p 309, Figure 5b, 5d.

Figure 1.1. Development of lysigenous aerenchyma in the root cortical tissue of cotton grass. Upper photo shows a root lacking aerenchyma. Lower photo shows the breakdown of cortical cells. From Gaynard and Armstrong (1987).

around the root (Jackson and Drew 1984).

In roots that lack appreciable aerenchyma, there exists an oxygen gradient within the root. The central core of the root tissue is significantly more hypoxic than the cortex, and the cortex more hypoxic than the epidermis (Drew 1990). Plants that have significant aerenchyma will not likely contain an anoxic gradient such as this if the upper leaves are in contact with the air, as the cortex would be the most oxygenated tissue. The loss of cortical cells during the production of aerenchyma does not seem to impair radial movement of solutes into and out of the root (Drew and Saker 1986).

The organs of wetland species, such as rice, form aerenchyma even when well-aerated, indicating that its formation is constitutive. In most dryland species, however, aerenchyma develops in oxygen stressed tissues only, and its formation is greatly stimulated by ethylene, as well as nitrogen and phosphate (Drew et al. 1989). Compared to tissues without aerenchyma, waterlogged maize and wheat roots with well developed aerenchyma resulted in higher oxygen and ATP concentrations up to 300 mm from the aerobic shoots where the oxygen originates (Drew et al. 1985, Thompson et al. 1990). Aerenchyma in maize, in contrast to rice, does not extend into the apical zone of the roots, and therefore the respiration rates in these tissues are greatly reduced (Drew and Stolzy 1991). A positive correlation between the amount of aerenchyma and enhanced growth in an hypoxic environment was shown by Justin and Armstrong (1987) in their study of 91 tolerant and intolerant plant species.

Other structural responses to hypoxia found in tolerant species involve the suberization and lignification of cell walls just beneath the epidermis (Clark and Harris 1981, Armstrong and Armstrong 1988). This adaptation is assumed to prevent radial diffusion of oxygen out of the root, and thus provide for better aeration of meristematic zones where oxygen consumption is the greatest (Armstrong 1971, 1978). In addition, the development of highly aerenchymatous adventitious (lateral) roots is also common in response to low oxygen concentrations. These roots are produced very near the surface of the soil in an attempt to gain access to oxygen in the thin oxygenated layer of the soil, and also to replace many functions of the original, damaged root system (Jackson and Drew 1984). In woody species, pore-like lenticels can develop at the stem base to improve gas exchange into the roots (Hook 1984).

1.4 Cytological Responses to Flooding

The decrease in ATP production during hypoxia results in membrane phospholipid deterioration, causing the disruption of cell and organellar membranes (Hochochka 1986). Vartapetian et al. (1987) and Vartapetian (1991) showed that all organs of flood sensitive plants (pea, wheat and maize) exposed to anoxic conditions for 30-90 min showed a swelling of mitochondria and a decrease in mitochondrial cristae. Upon longer exposure to hypoxia, the swelling reverted back to normal and mitochondrial cristae could form, however, after about 6 to 9 h of hypoxia, irreversible destruction of mitochondria and other organelles occurred. Vartapetian et al (1987) found that this pattern corresponded to the energy charge (see Section 1.5) of the cell, as glycolytic production of ATP was probably insufficient for the proper formation and maintainance of mitochondria immediately after the imposition of the stress. After 1 h, the energy charge of the cell increased and was sufficient for the maintainance of mitochondria. After 6 to 9 h, the cell could no longer provide the energy needed for cell integrity, and the mitochondria and other organelles disintegrated.

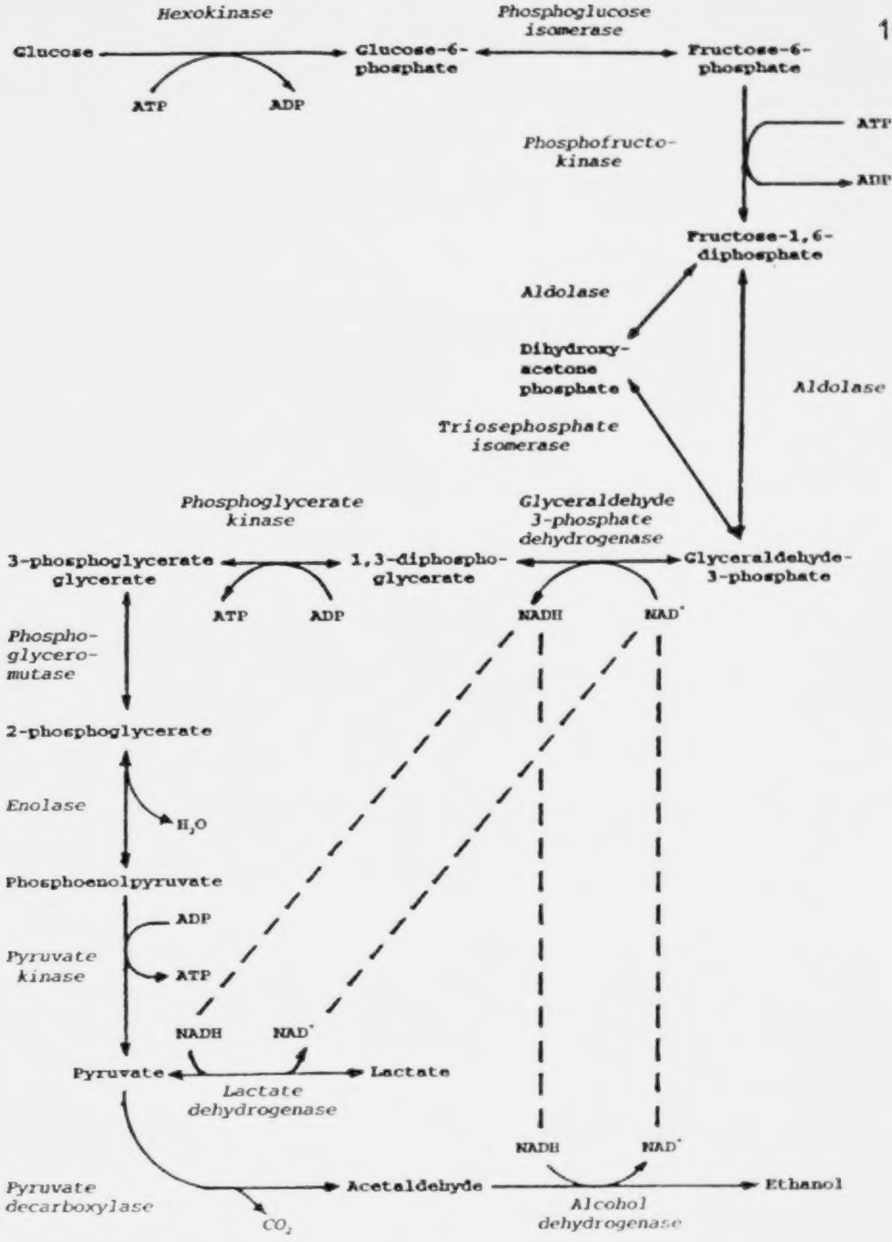
In the experiments where excised roots of flood tolerant and susceptible species were supplied with 3% glucose in the flooding solution, mitochondria were significantly better preserved during hypoxia as compared to the mitochondria of cells not supplied with glucose (Webb and Armstrong 1983). Studies on the ultrastructure of rice coleoptiles from intact plants grown hypoxically show that the lipid composition was similar to aerobic coleoptiles, and that hypoxically assembled mitochondria possessed intact membranes (Vartapetian et al. 1978).

Aldrich et al. (1985) studied the cytological events in maize roots exposed to 26 h of hypoxic treatment, and showed that chromatin organization differed drastically under oxygen stress conditions. Between 8 and 14 h of hypoxic stress, chromatin in interphase nuclei was highly condensed, and after 24 h, it partially dispersed again. Since strongly condensed chromatin ordinarily suggests reduced transcriptional activity, these authors suggested that the reduced protein synthesis which occurs under hypoxic conditions is consistent with these cytological observations. Cell division is also reduced by hypoxic stress, as Williamson (1968) observed the inhibition of mitosis in *Vicia faba* cells during hypoxia.

1.5 Metabolic Responses to Flooding

During hypoxic stress, the transfer of electrons from cytochrome oxidase to oxygen is reduced, and the electron transport chain and oxidative phosphorylation are blocked. The reduced nicotinamide adenine dinucleotide (NADH) and flavoproteins produced by glycolysis and the TCA cycle are no longer consumed, and the TCA cycle is blocked by feedback inhibition. Glycolysis can continue, however, provided that the NADH that is generated during the process can be oxidized.

The efficiency of glycolytic phosphorylation is low compared to that of oxidative phosphorylation. The consumption of one mole of glucose during glycolysis results in a net production of 2 moles of ATP, compared to 38 moles of ATP produced by oxidative phosphorylation. The regeneration of NAD^+ necessary for continued glycolysis can be provided by the production of ethanol and lactate (Fig. 1.2). In several plant species, an increase in the rate of hypoxic glycolysis (the Pasteur Effect) has been shown, and it is thought that this response meets the cell's ATP requirements until morphological changes such as aerenchyma and/or adventitious roots can relieve the tissue of hypoxia (Jackson and Drew 1984).



Glucose + 2 P_i + 2 ADP → 2 ethanol + 2 CO_2 + 2 ATP + 2 H_2O

Glucose + 2 P_i + 2 ADP → 2 lactate + 2 ATP + 2 H_2O

Figure 1.2 Anaerobic glycolysis resulting in lactate and ethanol as end-products. Modified from Stryer (1981).

All plant species appear to possess the necessary biochemical machinery to allow for at least a few hours of survival in oxygen-free environments. In metabolically active maize root tips devoid of oxygen, the ATP present within the cell would only last 1 to 2 min without its further synthesis (Roberts et al. 1984a). These organs, however, can survive greater than one day of hypoxia, since there is a regeneration of ATP primarily through ethanolic glycolysis. Some wetland species can thrive without oxygen, and can survive these conditions for over a week until structural and anatomical adaptations can restore oxygen availability. Other wetland species are no more biochemically tolerant to hypoxia than pea, and therefore, must depend on their structural and anatomical characteristics to obtain oxygen immediately upon flooding (Jenkin and ap Rees 1983).

Hypoxic conditions generally result in a decrease in cellular ATP concentrations, and some researchers have used the energy status of the cell as a measure of flood tolerance. The energy status of a cell is estimated by the adenylate energy charge (AEC) and is determined by the equation:

$$\mathrm{AEC} = \frac{([\mathrm{ATP}] + 0.5\,[\mathrm{ADP}])}{([\mathrm{ATP}] + [\mathrm{ADP}] + [\mathrm{AMP}])}$$

A value of 1.0 is obtained when only ATP is present, and zero when only AMP is present. The AEC of aerobic cells is generally stabilized between 0.8 and 0.95, but under hypoxic conditions it immediately falls (Jackson and Drew 1984). In excised maize root tips, the AEC value drops to 0.2 under anoxic conditions. The addition of exogenous glucose to anoxic root tips results in an increase in AEC to 0.6 (Saglio et al 1980), showing that the addition of a useable carbon source results in an increase of glycolysis. In rice, the AEC dropped immediately in coleoptiles upon exposure to hypoxia (from 0.9 to 0.5, Mocquot et al. 1981), but in the following 24 h, increased to 0.75 to 0.85 and remained stable for days while protein, DNA and RNA synthesis remained active (Mocquot et al. 1977). The unusual ability of rice coleoptiles to transport carbohydrate from the seed during hypoxia is probably important for this increase in energy charge.

Hochachka (1986) suggested that for cells to sustain periods of hypoxic stress, they must satisfy several fundamental requirements of metabolism including: maintenance of energy production through ATP synthesis; oxidation of NADH in the absence of oxygen as a terminal electron acceptor; avoidance of the toxic effects of undesirable end products; continuity of membrane integrity; and conservation of fermentable substrates.

1.5.1 Crawford's Metabolic Theory of Flooding Tolerance

The production of ethanol in hypoxic plant tissues is generally quite high, and since this pathway would result in the net synthesis of ATP and the regeneration of NAD^+, then ethanol production would seem able to resolve the problems of hypoxia. However, arguments have been made in the past that suggest flooding injury is due to the phytotoxic effects of metabolic end-products such as ethanol (Fulton and Erickson 1964, Kramer 1951). Crawford (1967) and McManmon and Crawford

(1971) studied several flood tolerant and intolerant plant species for alcohol dehydrogenase (ADH) activity and ethanol production, and found that flood tolerant plants produced decreased amounts of ADH. They proposed that flood-tolerant plants were successful because they could avoid the toxic effects of ethanol. McManmon and Crawford (1971) then proposed a "metabolic theory of flood tolerance" based on these studies (Figure 1.3). In it, they stated that tolerant species did not produce large amounts of ethanol, nor did they increase the rate of glycolysis. As an alternative, these plants accumulated large amounts of malate, a compound that would be non-toxic to the cell. Malate would be produced by the chemical reduction of the oxaloacetate derived from phosphoenolpyruvate carboxylation. Flood-intolerant species, on the other hand, produced large amounts of ethanol and had a very difficult time surviving its toxic effects. Although NAD^+ is regenerated, the drawback of this model is that malate synthesis would not result in a net synthesis of ATP. Since that time, most of the major tenets of this theory have been shown to be incorrect, presumably due to the small number of plant species studied in the original experiments.

Accelerated glycolysis and ethanolic fermentation is now known to be active in virtually all "flood tolerant" as well as "non-tolerant" species growing under hypoxic conditions (Jackson and Drew 1984 and references within). In addition, the idea that ethanol toxicity occurs at physiological concentrations has been refuted in many plant species (Alpi et al. 1985, Jackson et al. 1982, Rumpho and Kennedy 1983). Plants have the ability to excrete ethanol to the aqueous environment or

This figure has been removed because of copyright restrictions. The figure that was to be presented here is found in:

> Jackson MB and Drew MC (1984) Effects of flooding on growth and metabolism of herbaceous plants. *In* Kozlowski TT, ed, Flooding and Plant Growth, Academic Press, Orlando, FL, Figure 8, p. 77.

Figure 1.3 Possible metabolic difference between flood-tolerant and -intolerant species. PEPC, phosphoenolpyruvate carboxylase; MDH, malate dehydrogenase. From Jackson and Drew (1984).

transport it from poorly aerated parts of the plant to well oxygenated areas where it is metabolized (Fulton and Erickson 1964, Rumpho and Kennedy 1983). Although the original theory of flood tolerance has for the most part been refuted, it did provide much of the impetus for future research on other mechanisms of flood tolerance.

1.5.2 Adh Mutants and Flood Tolerance

All plant species appear to produce ethanol upon exposure to hypoxic stress, and the essential role of ADH in flood tolerance was shown in maize *Adh1*$^-$ mutants. *Adh1*$^-$ seeds could not germinate anaerobically, and seedling survival was greatly reduced to only a few hours under hypoxic conditions (Harberd and Edwards 1982, Lemke-Keyes and Sachs 1989a, Roberts et al. 1984a, 1984b, Schwartz 1969). The cytoplasmic pH in root tips of these *Adh1*$^-$ plants decreased much more rapidly than wild type, and cell death occurred much faster (Roberts et al. 1984b). The activity of ADH2 in these *Adh1*$^-$ plants was insufficient to sustain normal ethanol production and cytoplasmic pH regulation. These data strongly suggest that ethanol production by ADH plays a major role in flood- tolerance. Lemke-Keys and Sachs (1989a), however, studied nine maize lines which differed in hypoxia tolerance and found that ADH activity was not correlated to the degree of flood tolerance of these lines. This indicated that ADH production has a role in short-term tolerance to flooding only and other factors are important for tolerance over an extended period of time.

Lemke-Keys and Sachs (1989b) analyzed genetically a mutation which allowed *Adh1*$^{-}$ lines of maize to survive hypoxic treatment. This mutation segregated as a single recessive gene, and was shown not to be a revertant of the *Adh1*$^{-}$ mutation, as ADH activities remained low in these plants. This line, named anaerobic tolerant null (ATN), did not restore wild-type tolerance to hypoxia, as these plants did not survive as long as wild type plants. The ATN mutation does not affect the activity of lactate dehydrogenase (LDH) or pyruvate decarboxylase (PDC), and it has no effect on flood tolerance in an *Adh1*$^{+}$ background (MM Sachs, personal communication). It would be interesting to know how this gene partially restores flood tolerance.

1.5.3 The Davies/Roberts pH Stat Hypothesis

Davies (1980) proposed a model for the short-term tolerance of flooding which involves the pH of the cell regulating the synthesis of ethanol, a product which is important in the survival of the cell under hypoxic conditions. Initially, the synthesis of lactate from pyruvate (Figure 1.2) would lower the pH in the cytoplasm, this would activate PDC and ADH since they function optimally at a low cytoplasmic pH. LDH activity is highest at an alkaline pH, and would be reduced as the pH dropped, and lactate would no longer be produced. At the same time ethanol production would increase. Therefore, after the initial pH drop within the cell, the change in the activities of these enzymes would maintain a constant pH.

Non-invasive NMR data in maize supported this theory, showing that the pH in the cytoplasm of root cells decreased from 7.3 to 6.8 during the first 20 min of hypoxia, presumably due to the accumulation of lactate, and then leveled off after this time as lactate synthesis was halted (Roberts et al. 1984a). The vacuolar pH, however, remained relatively stable at pH 5.6, as a cytoplasmic/vacuolar gradient was maintained. After about 10 h, maize root cells are no longer able to maintain this vacuolar gradient, presumably due to a deficiency in ATP production. These researchers hypothesized that the leakage of protons from the vacuole is a major contributor to cell death. Saint-ges et al. (1991) later found that the initial drop in cytoplasmic pH did not match the increase in lactate concentration, and therefore questioned the hypothesis that lactate synthesis triggered ethanolic glycolysis. They stated that other species (eg. rice and yeast) also showed an immediate drop in pH, however, very little lactate was produced in these organisms under hypoxic conditions.

The drop in cytoplasmic pH is not universal, as different tissue types and different plant species vary in cellular pH in hypoxic environments. Menegus et al. (1989, 1991) have shown that shoots of flood-sensitive plants, such as wheat, exhibit a large drop in cytoplasmic pH, whereas tolerant species (rice and *Echinochloa*) show an initial, smaller drop in pH, followed by an alkalinization of the cytoplasm. In tissues that underwent this alkalinization, ethanol production and ADH activity were high. This refutes the pH stat theory which predicts low ADH activity at a higher pH.

It is apparent that cytoplasmic acidification is correlated with flood-intolerance and may be an early indicator of intolerance. It may, however, be a consequence of cell death rather than a cause. If causative in nature, this acidification may result from the accumulation of acidic end-products (eg. lactate) produced by non-tolerant species, or due to the depressed energy status of the cell which may be insufficient to maintain the energy dependent pH gradient between the acidic vacuole and alkaline cytoplasm. Roberts et al. (1984a) proposed that plants can survive longer periods without oxygen than animals because they have the ability to produce ethanol. They stated that animal cells quickly undergo cellular acidosis due to the production of large quantities of lactate. Ethanol is a difficult molecule to regulate intracellularly and intercellularly, as it moves passively across membranes. Plants, however, can easily excrete it into the surrounding medium or transport it to the leaves where it is transpired or oxidized.

1.5.4 Alternate Pathways of Fermentative Respiration

Although hypoxic ethanol production is virtually universal in plants, it alone is not likely to confer long-term biochemical flood-tolerance to plants. Glycolysis coupled with another pathway of hypoxic metabolism may enable roots of tolerant species to produce more energy per mole of glucose, or produce a compound which is important in cytoplasmic pH regulation. These factors may enable extended survival compared to susceptible plants which are capable only of ethanolic fermentation.

Pathways of hypoxic metabolism other than ethanolic fermentation have been well documented in many other organisms, and these pathways are thought to contribute significantly to energy production in hypoxia tolerant organisms such as bacteria, oysters, parasitic helminths and deep-sea animals (Hochochka 1986). Substrate level phosphorylation by a partial TCA cycle is used by invertebrate organisms, replacing the usual aerobic pathways. Recently, several of these pathways have been investigated to determine whether they have a role in conferring tolerance to flooding in plants. Some plants are known to accumulate succinate and alanine upon flooding (Effer and Ranson 1967, Menegus et al. 1989, 1991, Streeter and Thompson 1972). These pathways have been studied in some detail recently, and models of their function have been put forward. In addition to producing ATP, the increase in levels of these so-called alternate end-products may also permit the reoxidation of NAD(P)H.

Vanlerberghe et al. (1990a) studied anaerobic metabolism in the single-celled algal species *Selanastrum minutum* and, based on their results, came up with a model for the function of a partial reductive TCA cycle (Figure 1.4). In their model, alanine accumulates in the short-term by transamination of pyruvate by glutamate (Figure 1.4a). Oxaloacetate would be produced from these reactions, and would eventually be reduced to succinate through a partial TCA cycle. Coupling the production of succinate to glycolysis would double the ATP produced by glycolysis (net 4 ATP), and the reduction and oxidation reactions of NADH would be balanced. After the available aspartate is consumed, phosphoenolpyruvate would be

This figure has been removed because of copyright restrictions. The figure that was to be presented here is found in:

Vanlerberghe GC, Feil R and Turpin DH (1990) Anaerobic metabolism in the N-limited alga *Selenastrum minutum*. I. Regulation of carbon metabolism and succinate as a fermentation product. Plant Physiol 95: 1116-1123, Fig. 8.

Figure 1.4. Proposed pathways for the hypoxic production of succinate in the alga, *Selanastrum minutum*. A) In the short-term, aspartate provides the carbon skeleton for oxaloacetate production. This pathway results in a 1:1 relationship between the production of succinate and alanine. The consumption of aspartate continues until the available aspartate is depleted. b) the long-term pathway occurs upon depletion of aspartate. Abbreviations: TP; triose phoshate; PEP, phosphenolpyruvate; Pyr, pyruvate; OAA, oxaloacetate; 2-OG, 2-oxoglutarate; Glu, glutamate. From Vanlerberghe et al. (1990a).

converted to oxaloacetate directly, thus bypassing pyruvate synthesis, and completing the production of succinate (Figure 1.4b). This longer-term pathway would generate only 2 ATP, and therefore would provide no more energy than that of ethanol or lactate synthesis. Succinate, however, would be retained within the cell (unlike ethanol) and may be less toxic than lactate.

The short-term production of alanine is accounted for in the model of Vanlerberghe et al. (1990a, 1991), however, alanine synthesis is known to increase over several days of hypoxia in many plant species (Vanlerberghe et al. 1991b and references therein). Using radioactive NH_4^+, Vanlerberghe et al. (1991b) and Menegus et al. (1993) found that when ammonium ions were assimilated by hypoxic cells of *Selanastrum minutum* and rice, respectively, virtually all of the labelled nitrogen was extracted from the cells as alanine. The uptake of ammonium ions and the synthesis of alanine could maintain energy and redox balance within the cell, at the same time regenerating glutamate and 2-oxoglutarate (Figure 1.5). The possible role of alanine synthesis in this pathway would be to remove the toxic effects of ammonium ions that accumulate in the soil under hypoxic conditions, and to provide an energy efficient mechanism of hypoxic nitrogen assimilation for the rapid production of amino acids upon return to aerobic conditions. Menegus (1993) speculated that alanine accumulation in rice coleoptiles also plays a role in osmotic pressure generation, allowing the rapid uptake of water necessary for the expansion of cells which is important for the extension of the coleoptile. This growth would

This figure has been removed because of copyright restrictions. The figure that was to be presented here is found in:

Vanlerberghe GC and Turpin DH (1990) Anaerobic metabolism in the N-limited green alga *Selanastrum minutum*. II. Assimilation of ammonium by anaerobic cells. Plant Physiol: 1124-1138, Fig. 8.

Figure 1.5. Proposed pathway of *Selanastrum minutum* assimilating ammonium ions and producing alanine under anaerobic conditions. Abbreviations: TP, triose phosphate; PEP, phosphoenol pyruvate; Pyr, pyruvate; Gln glutamine; Glu, glutamate; 2-OG, 2-oxoglutarate. From Vanlerberghe and Turpin (1990b).

allow the coleoptile to rapidly enter aerobic zones, and allow the transport of oxygen to other parts of the plant.

1.6 Hypoxic Gene Expression

Hypoxic treatment results in a very dramatic shift in the synthesis of plant proteins, the function of which is to override normal carbohydrate metabolism and interrupt the normal developmental program of gene expression. Much of the research on the anaerobic response and hypoxic gene expression has been done on maize, specifically maize *Adh1*, and therefore much of this section will be devoted to this work.

1.6.1 The Anaerobic Proteins

In a similar fashion to that of heat shock (Cooper and Ho 1983), hypoxically stressed maize seminal roots show a repression of pre-existing protein synthesis and a *de novo* synthesis of a new set of proteins, the anaerobic proteins (ANPs). In the first hour of hypoxic stress, there is a rapid synthesis of a 33 kD class of stable proteins referred to as the transition proteins (TPs, Sachs et al. 1980). During the second hour, the synthesis of an additional 20 or so proteins is initiated, which accounts for >70% of the protein synthesized after 5 h of hypoxia. After 72 h, protein synthesis is reduced as cell death occurs (Sachs et al. 1980). The degradation of aerobic mRNA is not immediate upon exposure to hypoxic stress, as a mechanism of selective translation of newly synthesized hypoxic mRNA prevents the translation of these aerobic messages. However, after exposure of the roots to 24 h of hypoxic stress, the aerobic mRNAs are completely degraded (Sachs et al. 1980). The anaerobic response is not as rapid as the heat shock response in plants, and all but one of the ANPs appear to be a completely different set of polypeptides from the heat shock proteins (Kelley and Freeling 1982).

The identities of the TPs are not yet known, although several of the other ANPs have been identified, including ADH1 and ADH2 (Ferl et al. 1979, Sachs and Freeling 1978), sucrose synthase (Springer et al 1986), glucose-6-phosphate isomerase (Kelley and Freeling 1984a), aldolase (Kelley and Freeling 1984b), PDC (Wignarajah and Greenway 1976), phosphoglucomutase (Bailey-Serres at al. 1988), phosphoglycerate mutase (Bailey-Serres et al. 1988), enolase (Bailey-Serres et al. 1988), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Russell and Sachs 1989), LDH (Hanson and Jacobson 1984, EM Christopher and AG Good, unpublished data), and alanine aminotransferase (AlaAT, Good and Crosby 1989). All of these enzymes are classified as glucose metabolizing, glycolytic or fermentative enzymes (Fig. 1.1); AlaAT is the only known amino acid biosynthetic ANP (Olive et al. 1991).

The maize anaerobic response operates in tissues other than roots, as the coleoptile, mesocotyl, endosperm, scutellum and anther wall selectively synthesize the ANPs in a fashion virtually identical to that of roots (Okimoto et al. 1980). Maize leaf tissue, however, does not synthesize ANPs and does not survive even brief exposures of hypoxic stress (Okimoto et al. 1980).

This shift in hypoxic protein synthesis has been seen in the root tissue of many plant species including rice, soybean, sorghum, barley, pea and carrot (Sachs et al. 1980). In soybean, 15 min of hypoxia resulted in the dissociation of polyribosomes and the inhibition of normal protein synthesis. There is a rapid reformation of polyribosomes upon aeration following 5 h of hypoxic stress, even in the absence of new RNA synthesis (Lin and Key 1967). The ANP profile of soybean is comprised of only four polypeptides, one of which is ADH. The relative sensitivity of soybean to hypoxia compared to maize may be a result of the reduced ANP synthesis in soybean (Russell et al. 1990). Rice and barnyard grass (*Echinochloa* spp.) do not undergo the complete dissociation of polysomes under anoxic conditions (Kennedy et al. 1992). Barnyard grass shows an initial period when ANPs are synthesized, but within 24 h of the onset of the stress, aerobic protein synthesis resumes (Kennedy et al. 1992). In rice seedlings, several proteins are synthesized under hypoxic conditions, however, the synthesis of these proteins decreases with time (Mocquot et al. 1981). Thus, hypoxic expression of proteins in the flood tolerant (rice and barnyard grass) and flood susceptible (maize) species studied to date are quite different, although increased ADH activity is common to them all.

1.6.2 ANP Expression Patterns

Hypoxic treatment causes ADH activity to increase 5-fold (or greater) in the roots of maize seedlings, however, ADH is expressed in several other tissues as well, in either a constitutive or inducible fashion (Bailey-Serres et al. 1988). Two *Adh* genes exist in maize, and both are equally inducible under hypoxia. Transcriptional induction of *Adh1* and *Adh2* is detectable within 1 h of the start of hypoxic stress, and mRNA levels increase 20-50 fold over aerobic levels after about 5 h of the stress (Gerlach et al. 1982, Rowland and Strommer 1986). This level of expression is maintained up to 48 h for *Adh1* and 10 h for *Adh2*, before the levels begin to decline (Dennis et al. 1985). Rowland and Strommer (1986) performed run-off transcription assays, and demonstrated that the increases of *Adh1* mRNA levels are due to an increase in transcription rate, although there is some evidence for increased stability of the *Adh1* transcript in hypoxic versus aerobic conditions. Upon return to oxygenated conditions, *Adh1* transcription is virtually undetectable within 1 h, although *Adh1* mRNA can be detected for at least another 26 h after return to oxygenation.

The expression patterns of the other ANPs has also been characterized at the level of enzyme activity, and in some cases at the level of mRNA (Table 1.1). There is a large range in the degree of induction of these different ANPs and their mRNAs. The induction of ANP mRNA generally occurs within the first 30 min to 3 h and peaks between 2 and 10 h (Russell and Sachs 1989).

Table 1.1. Enzyme activity and mRNA induction of the known ANPs. All values are from maize unless otherwise noted. The enzyme induction values are of total cellular activity, and therefore may include the activities of uninduced isoforms. Activity increases listed as "inducible" have been shown to increase in response to hypoxia, however quantitative values were not listed in the original refernces. ND, not determined.

Enzyme	Activity Increase (fold)	Peak mRNA Level and Time of Peak	References
Alcohol Dehydrogenase 1 and 2	10	20 to 50-fold in 5 to 10 hrs (both Adh1 and Adh2)	Freeling (1973) Gerlach et al. (1982) Hageman and Flesher (1960) Rowland and Strommer (1986)
Alanine Amino-transferase	>2 (maize) 4 (barley)	ND	Good and Crosby (1989b)
Aldolase	2	20-fold in 2-3 hrs	Dennis et al. (1987) Hake et al. (1985) Kelley and Freeling (1984a, 1984b)
Enolase	2	5-fold in >48 hrs	Lal et al. (1991)
Glyceraldehyde 3-Phosphate Dehydrogenase	1.5	6-fold in 6 hrs	Bailley-Serres et al. (1988)
Lactate Dehydrogenase	20 (barley) 3.5 (maize)	8.5-fold (barley) in <48 hrs	Hoffman et al. (1986) Hondred and Hanson (1990) Christopher and Good (unpub.)
Phospho-glucomutase	inducible	ND	Bailley-Serres et al. (1988)
Phospho-glucoisomerase	2	ND	Kelley and Freeling (1984a)
Pyruvate Decarboxylase	5	20-fold in 24 hrs	Kelley (1989) Lazlo and St. Lawrence (1983) Wignarajah and Greenway (1976)
Sucrose Synthase I	0	20-fold in 24 hrs	Russell and Sachs (1989) Springer et al. (1986)

1.6.3 Transcriptional Control of ANP Synthesis

Several genes encoding ANPs of maize have been isolated and sequenced, and in some cases the upstream regulatory regions have been dissected to identify functional elements. Transient expression assays were initiated using the *Adh1* regulatory region linked to either chloramphenicolacetyl-transferase (CAT) or B-glucuronidase (GUS). These constructs were electroporated into maize protoplasts. The activity of the marker gene was then assayed after the cells have been exposed to a period of hypoxic stress (Howard et al. 1987). Deletion and linker scanning mutagenesis of the regulatory region and subsequent transient expression analysis has identified two sub-regions that are essential for hypoxic induction (Walker et al. 1987). These regions (-140 to -124 and -113 to -99) were separated by a 10 bp segment (-123 to -114), and together were designated the anaerobic regulatory element (ARE). The orientation and position of the ARE did not affect its ability to drive transcription, and when multiple ARE sequences are juxtaposed upstream of a reporter gene, the amount of expression is additive (Bailey-Serres et al. 1987, Olive et al. 1991). This indicated that the ARE functioned as an enhancer element in the expression of the *Adh1* gene. Mutational analysis to determine the functional sequences in the *Adh2* upstream region has not been reported.

Sequence analysis of the upstream regulatory regions of*Adh2* and other ANP genes from several species identified regions of similarity to the ARE of *Adh1* (Fig. 1.6). Each of these promoters contains a core sequence motif (TGGTTT), although in the case of pea it is present in the reverse orientation. Functional analysis of the

This figure has been removed because of copyright restrictions. The figure that was to be presented here is found in:

Olive M, Karambin S, Ellis JG, Llewellyn D, Peacock WJ, Dennis ES (1991) The anaerobic responsive element. *In* Plant Molecular Biology 2, RG Hermann and B Larkins, eds, Plenum Press, NY, pp 673-684, Fig. 6.

Figure 1.6. Sequences conserved in the upstream regulatory region of anaerobically inducible genes. The positions of bases are given relative to the transcription start site. The ARE core is underlined in each case. From Olive et al. (1991).

maize aldolase and pea *Adh* genes by deletion of regions upstream of the gene identified the ARE sequence within a region necessary for hypoxic expression (Dennis et al. 1988, Olive et al. 1991). The only deviation from the core sequence is found in ARE subregion I of the *Adh1* gene, where a cytosine residue replaces a thymidine residue at the first position (Fig. 1.6).

The essential role of the ARE was supported by the discovery that nuclear proteins bind to these sites, as identified by DNase and in vivo DMS footprinting techniques (Ferl and Nick 1987). Although the *Adh1* and *Adh2* genes are similarly induced under hypoxic conditions in maize roots and cultured cell lines, *in vivo* footprinting indicates that trans-acting factors bind the two AREs in a dissimilar fashion (Paul and Ferl 1991). The *Adh1* gene has two types of DNA binding factors associated with the promoter. The first is constitutively bound to the ARE, whereas two additional factors bind only under hypoxic conditions. In contrast, all of the factors that bind to the *Adh2* promoter are constitutive, and not affected by hypoxia (Paul and Ferl 1991).

1.7 Plant Alanine Aminotransferases

AlaAT reversibly catalyzes the production of alanine and 2-oxoglutarate from pyruvate and glutamate (Figure 1.4). In plants, AlaAT has a role in several metabolic processes, including amino acid biosynthesis, gluconeogenesis, and some forms of C_4 photosynthesis (Watson et al. 1992). Despite its importance, this enzyme has received little attention until recently. Early studies showed that AlaAT activity in green leaves of several plants can be found primarily in the peroxisomes (90% of activity) and in the mitochondria (10%), although in non-green tissues AlaAT was primarily located in the mitochondria. It was also shown that AlaAT had glutamate:glyoxylate aminotransferase activity as well (Biekmann and Feierabend 1982, Noguchi and Hayasha 1981). This was not unusual, as aminotransferases often have more than one aminotransferase activity (Givan 1980).

Recently, AlaAT has been purified to homogeneity from *Chlamydomonas reinhardtii* (Lain-Guelbenzu et al. 1991) and from green leaves of broomcorn millet (*Panicum miliaceum*, Son et al. 1991). In *Chlamydomonas* and *P. miliaceum*, AlaAT is homodimer composed of 45 and 50 kD subunits, respectively. In *P. miliaceum* leaves, AlaAT functions in the transfer of C_3 units between the cytosol of bundle sheath and mesophyll cells to maintain a nitrogen-carbon balance in the C_4 NAD-malic enzyme type of photosynthesis (Hatch 1987). This enzyme increases ~50-fold in etiolated leaves exposed to 5 d of light, and is also induced in green leaves of *P. miliaceum* upon recovery from nitrogen stress (Son et al. 1992). AlaAT is present in the cytosol of leaf bundle sheath and mesophyll cells of *P. miliaceum* (Hatch 1987, Son et al. 1991). Three isoforms of leaf AlaAT are visible on non-denaturing gels, AlaAT-2 being the light inducible isoform. Immunoblots of SDS-PAGE and non-denaturing gels, however, identify only a single band (Son et al. 1992), indicating that the polyclonal antibody raised to AlaAT-2 is specific to that isoform. A cDNA encoding the light inducible AlaAT-2 from *P. miliaceum* has been isolated and sequenced.

Spectrophotometric analysis has demonstrated that AlaAT activity increases 4-fold in barley roots exposed to hypoxic conditions for 5 d, although the rate of activity increase in several other cereals was generally not as high as that of barley (Good and Crosby 1989). This increase seen when measured spectrophotometrically, however, was not visible as an increase in the AlaAT activity band intensity on non-denaturing gels in either barley or maize (Good and Crosby 1989, Watson et al. 1992), thus causing some uncertainty as to the hypoxic induction of activity of this enzyme.

1.8 Goals of this Thesis

The aim of this thesis was to characterize the expression patterns of the hypoxically inducible root AlaAT, and to obtain a better understanding of the adaptive role of AlaAT and alanine synthesis in a plant's response to periods of long-term hypoxic stress. Specific objectives were to:

1) Identify, purify and study the biochemical characteristics of the hypoxically inducible barley root AlaAT as an initial step in determining the regulation of its expression.

2) Determine if the hypoxic increase of AlaAT activity was regulated in a similar fashion to that of the other known ANPs. This was accomplished by isolating and

sequencing the corresponding AlaAT cDNA and raising antibodies to the purified AlaAT protein. These were then used as probes to determine the change in concentration of AlaAT mRNA and protein during hypoxic stress.

3) Quantitate the hypoxic accumulation of alanine in relation to other metabolic products of hypoxia in long-term experiments, to determine if these responses are similar to the well characterized short-term responses. In addition, the expression of several enzymes which synthesize some of these metabolites was examined, to see if their induction is necessary for metabolite accumulation. A representative flood-tolerant species (wild rice, *Zizania palustris* L.) and a relatively flood-intolerant species (barley, *Hordeum vulgare* L.) were studied for comparison purposes.

1.9 Bibliography

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2 Purification and characterization of a hypoxically inducible alanine aminotransferase from barley roots

2.1 Introduction

All the anaerobic proteins (ANPs) identified to date are involved in sucrose metabolism, glycolysis or fermentation. Recently, the activity of alanine aminotransferase (AlaAT) has been shown to increase several-fold in cereal roots exposed to hypoxic stress (Good and Crosby 1989b). AlaAT is the only known amino acid biosynthetic ANP, and based on enzyme activity, appears to be regulated in a pattern similar to ADH and LDH in maize and barley (Good and Crosby 1989a, 1989b, Hoffman et al. 1986, Lazlo and St. Lawrence 1983). AlaAT is a pyridoxyl-5-phosphate dependent enzyme, and catalyzes the production of alanine and 2-oxoglutarate from pyruvate and glutamate.

Although detailed examination of specific plant aminotransferases has been carried out in only a few cases, a couple of generalizations can be made. First, there are usually multiple isoforms of a specific aminotransferase within any plant tissue. Second, purified aminotransferases will often utilize a variety of substrates in catalyzing a transamination reaction (Hatch 1973, Hondred et al. 1985,

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A version of this chapter has been published. Good AG and Muench DG (1992). Plant Physiol 99: 1520-1525.

Wightman and Forest 1978). For example, Hatch (1973) identified two different aspartate aminotransferases and three alanine aminotransferases in leaves of *Atriplex spongiosa* using ion exchange chromatography. Lain-Guelbenzu et al. (1991) found that an alanine aminotransferase purified from *Chlamydomonas* also could utilize glyoxylate as a substrate in place of 2-oxoglutarate. However, Son et al. (1991) have purified AlaAT from broomcorn millet (*Panicum miliaceum* L.), and found that the enzyme was very substrate specific.

As a first step in understanding the hypoxic expression of AlaAT, this chapter describes the purification and characterization of the hypoxically inducible isoform of this enzyme.

2.2 Materials and Methods

2.2.1 Plant material and reagents

Barley (*Hordeum vulgare* L. cv Leduc) seeds were surface sterilized in 1% NaOCl, rinsed with water and planted in moist vermiculite. After 4-6 d growth at 20°C, individual seedlings were removed and inserted through holes in a foam stopper with the roots hanging below. Plants in their foam stoppers were placed in holes cut into a plexiglass board. This board fit atop a 20 L fish tank, so that the roots were hanging below. The tank and plexiglass lid were painted black to prevent light from entering. Plants were grown hydroponically with the roots submerged in aerated, half-strength Hoagland solution (See Appendix 1) for 15 to

25 d. Aerobic and hypoxic conditions were achieved by sparging the nutrient solution with air and N_2, respectively. Oxygen concentrations in the aerobic and hypoxic tanks were 0.26 and 0.0325 mM, respectively. Plants were grown under a 16 h photoperiod at 21•C and 250 µmol/m/s photosynthetic photon flux density supplied by white fluorescent lamps (Vita Lite, Dura Test Corp., Secaucus NJ, USA).

All of the chromatography materials were purchased from Pharmacia (Uppsala, Sweden). All amino acids, keto acids and enzymes were purchased from Sigma Biochemicals (St. Louis, Missouri). All other biochemicals were purchased from BDH Ltd. (Poole, U.K.).

2.2.2 Purification of AlaAT

AlaAT was purified from roots, with all steps being carried outat 4°C and all buffers containing 10 mM 2-mercaptoethanol. Hypoxically induced roots (100-200 g) were harvested, washed in distilled water and ground (1:2, w/v) in a blender in 50 mM Tris-HCl (pH 8.0, buffer A). Proteins were precipitated in the 35 to 65% (saturation) $(NH_4)_2SO_4$ fraction. The $(NH_4)_2SO_4$ precipitate was resuspended in buffer A and loaded as 12 mL samples on to a Superdex 26/60 G-200 gel filtration column (Pharmacia) that had been equilibrated with 50 mM Tris-HCl (pH 8.0), 150 mM NaCl. The column was washed and eluted at 3 mL/min using the same buffer. AlaAT activity eluted from this column as two peaks. The fractions from the second (hypoxically induced) activity peak (see "Results") were pooled and used for further purification. These fractions were dialysed overnight against two changes of buffer A and then loaded onto a 20 mL Q-Sepharose ion exchange column which had been equilibrated in buffer A. The column was then washed with 4 column volumes of buffer A at 1 mL/min, and the AlaAT activity was eluted at the same flow rate by applying a 0 to 0.5 M NaCl gradient in buffer A (4 column volumes). AlaAT activity eluted as a single peak from this, and all subsequent columns.

After dialysing the active fraction overnight against 25 mM Bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane (pH 7.1, buffer B), the sample was loaded on to a Mono-P chromatofocusing column (HR 5/5, Pharmacia) that had been equilibrated in buffer B. The column was then washed with 4 column volumes of buffer B and then eluted at 0.5 mL/min using a pH gradient of 7 to 5, which was generated using Polybuffer 74 as per the manufacturer's instructions. The fractions were collected into tubes containing 1/10 volume of 1 M Tris-HCl pH 8.0. This acted to readjust the pH of each fraction to between pH 7.4 and 7.9. The fractions containing AlaAT activity were diluted 3-fold in buffer A and loaded directly onto a Mono-Q column (HR 5/5, Pharmacia). This column was then washed with 20 mL buffer A at 1 mL/min and eluted using a 0 to 0.5 M NaCl gradient in 85 mL of buffer A at the same flow rate. The fractions containing AlaAT activity eluted at a salt concentration of approximately 160 mM NaCl and were stored in 10% (v/v) glycerol at -20°C. AlaAT activity had a half life of approximately 2 weeks at -20°C.

2.2.3 Assay of AlaAT activity and substrate specificity

AlaAT activity was assayed spectrophotometrically by monitoring NADH oxidation at 340 nm. Activity in the alanine → pyruvate direction was determined by coupling the reaction to NADH oxidation by LDH (Good and Crosby 1989b). The reaction mixture contained, in a final volume of 1 mL, 25 mM alanine, 5 mM 2-oxoglutarate, 0.1 mM NADH, 100 mM Tris-HCl (pH 8.0), 5 units of LDH (Sigma L2375) and 50 µL of extract. The reverse reaction was assayed in a mixture containing 6 units of glutamate dehydrogenase (Sigma G2501), 100 mM Tris-HCl (pH 8.0), 100 mM NH_4Cl, 0.1 mM NADH, 5 mM pyruvate and 5 mM glutamate. The K_m of alanine, 2-oxoglutarate, glutamate and pyruvate were determined by adding the appropriate concentration of substrate to the reaction mixtures above which lacked that substrate. The rate of decrease in absorbance at 340 nm was then recorded. Transamination between L-alanine and the keto acids 2-oxoglutarate or glyoxylate was assayed as above with the appropriate keto acid being used to start the reaction. All keto acids were supplied at a final concentration of 5 mM. Transamination between L-serine and the keto acid was assayed using the method of Nakamura and Tolbert (1983). The activity was measured spectrophotometrically in a mixture containing, in a final volume of 1 mL, 25 mM L-serine, 5 mM keto acid, 0.1 mM NADH, 0.1 M Tris-HCl (pH 8.0) and 1 unit of hydroxypyruvate reductase (Sigma G-5259). Transamination between L-aspartate and the keto acid was assayed spectrophotometrically as described by Hondred et al. (1985), in a mixture containing, in a final volume of 1 mL, 25 mM L-aspartate, 5 mM keto acid, 0.1 mM

NADH, 0.1 M Tris-HCl (pH 8.0) and 2 units of malate dehydrogenase (Sigma M-9004). All enzyme assays were linear with respect to time and enzyme concentration. One unit of AlaAT was defined as the amount catalyzing the formation of 1.0 µmol of product per min at 23°C

2.2.4 Inhibitors of AlaAT activity

Inhibition of AlaAT activity was assayed in the alanine → pyruvate direction as described above. Inhibitors were added in various concentrations to the assay mixture containing the purified enzyme but without 2-oxoglutarate and incubated at 4°C for 15-20 min. The reaction mixture was then assayed at 23°C for the nonspecific oxidation of NADH and the reaction started by adding 5 mM 2-oxoglutarate.

2.2.5 Other Assays

Protein concentration was determined by the Bradford assay (Bradford 1976) using BSA as a standard and measured spectrophotometrically at 590 nm. Column effluent was monitored spectrophotometrically for total protein by the absorbance at 280 nm.

2.2.6 Gel Electrophoresis

Non-denaturing gel electrophoresis was performed using 7% acrylamide mini slab gels. Samples were mixed in one-fifth volume gel loading buffer (65 mM Tris-

HCl [pH 6.5], 37.5 % glycerol, 0.1% [w/v] bromophenol blue) prior to loading. The running gel contained 7% acrylamide-0.8% bisacrylamide, 10% (w/v) sucrose, and 0.37 M Tris-HCl (pH 8.9), and was polymerized with 0.5 µL/mL TEMED and 0.32 mg/mL ammonium persulfate. The stacking gel consisted of 5% acrylamide-0.13% bisacrylamide, 10% (w/v) sucrose, 60 mM Tris-HCl (pH 6.8), 0.6 µL/mL TEMED and 16 µg/mL riboflavin, and was photopolymerized for 2 h. Gels were run at 4°C for about 3 h in running buffer (5 mM Tris, 38 mM glycine pH 8.3), and stained as described previously (Good and Crosby 1989b). Gels were incubated at 4°C for 30 min in prestain solution (50 mM Tris-HCl (pH 8.0), 20 units/mL LDH [Sigma L2375]). The solution was then supplemented with 30 mM L-alanine, 10 mM 2-oxoglutarate and 2 mM NADH. The gel was then incubated in this solution for 30 min at room temperature, and then rinsed with water for 10 min. The gels were then photographed on an ultraviolet light box. Isozyme bands were seen as a lack of fluorescence in regions where NADH is oxidized.

SDS-PAGE was performed using 8% (w/v) SDS-PAGE mini slab gels according to Laemmli (1970). The protein samples were denatured by adding 1 volume of denaturation buffer (0.5 M Tris-HCl [pH 6.8], 10% [v/v] glycerol, 10% SDS, 5% [v/v] 2-mercaptoethanol and 0.1% [v/v] bromophenol blue), and then boiled for 5 min. The running gel contained 8% acrylamide, 0.2% bis-acrylamide, 350 mM Tris-HCl (pH 8.7), 0.1% SDS, and polymerized by adding of 0.32 mg/mL ammonium persulphate, 0.5 µL/mL TEMED. The stacking gel contained 5% acrylamide, 0.13% bis-acrylamide, 110 mM Tris-HCl (pH 6.8), 0.1% SDS, and polymerized with 32 mg/mL ammonium persulphate and 0.5 μL/mL TEMED. Gels were run in 5 mM Tris-HCl, 192 mM glycine (pH 8.3) and 0.1 % (w/v) SDS, for approximately 2 h. The protein bands were visualized either by using Coomassie blue stain or a silver staining kit (Bio-Rad).

2.2.7 Molecular Mass Determinations

The apparent molecular mass of denatured AlaAT was determined by comparison of its mobility with that of known molecular mass standards on SDS-PAGE. The standards (Bio-Rad) consisted of phosphorylase B (97.4 kDa), bovine serum albumen (BSA, 66.2 kDa), ovalbumen (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). The apparent native molecular mass was estimated by FPLC using a Pharmacia Superose 6 column (HR 10/30, Pharmacia). Bio-Rad standards were used to calibrate the column and included thyroglobin (670 Kd), gammaglobin (158 Kd), ovalbumin (44 Kd), myoglobin (17 Kd) and vitamin B-12 (1.3 Kd). The column was equilibrated and eluted with 50 mM Tris-HCl (pH 8.0), 150 mM NaCl at a flow rate of 0.5 mL/min and using 100 μL sample volumes.

2.3 Results

2.3.1 Identification of the hypoxically induced AlaAT.

In order to purify the hypoxically induced AlaAT, we first characterized the number of isoforms present in roots, and then determined which of the isoforms was induced by hypoxia. Figure 2.1 illustrates the elution profile of AlaAT activity from the Superdex G-200 gel filtration column for both aerobic and hypoxic root tissue. AlaAT activity eluted as two distinct peaks, however the second peak contained the majority of activity and was the isoform that increased in total activity after hypoxic induction. During all subsequent purification steps, AlaAT activity eluted as a single peak from each column.

2.3.2 AlaAT Purification

The isolation of AlaAT from barley roots is summarized in Table 2.1 and Figure 2.2, A and B. While many purifications involve an ion exchange column prior to gel filtration, we found that the present order consistently gave both higher yields and better purification. The AlaAT activity eluted from the Q-Sepharose column as a single peak which was dialysed against Buffer B and loaded on to a Mono-P column. A comparison of the pH of the fractions containing AlaAT activity indicated that this isoform has a pI of approximately 5.7. The loss in enzyme activity during chromatofocusing occurred as a result of the low pH of the eluting buffer, rather than as a result of separation of two different isoforms, since no other peaks of activity were detected. In addition, when an aliquot of AlaAT activity from the previous step was adjusted in pH to 5.7, it lost its activity at a similar rate to the Mono-P purified fraction. The final purification step involved a high-resolution Mono-Q column. The AlaAT active fractions from this step showed a single

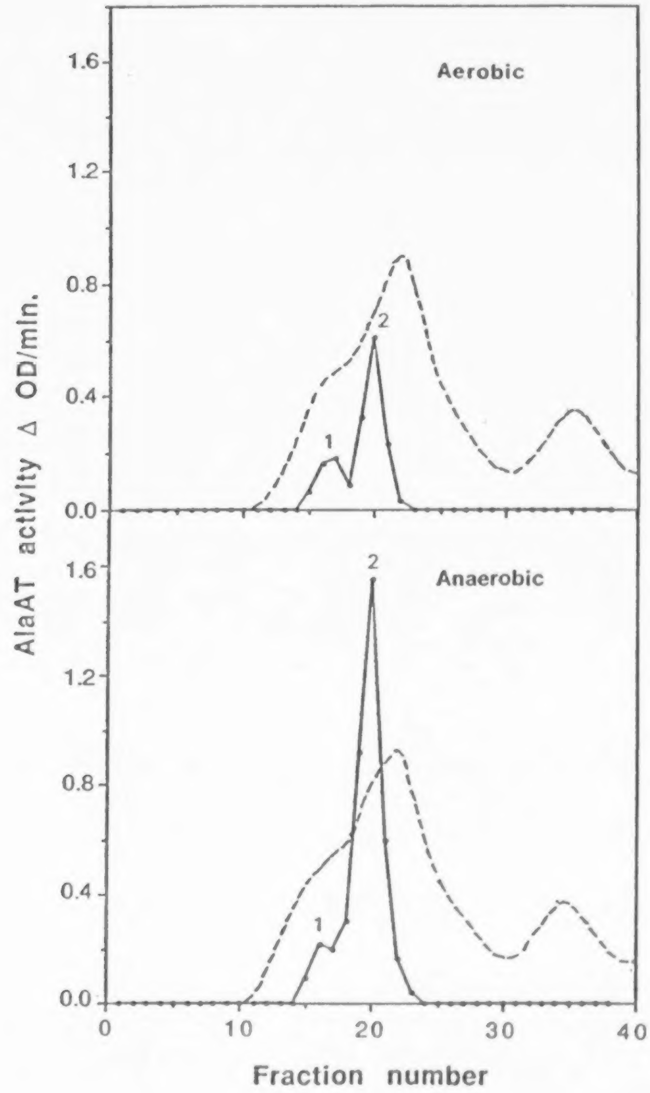

Figure 2.1. Gel filtration of AlaAT from aerobic and hypoxic barley roots on Superdex G-200. Details of the procedure are described in the text; (●) AlaAT activity; (---) total protein as determined by A_{280}. The fractions from the second peak (2) were pooled and used for further purification.

Table 2.1 Purification of AlaAT from hypoxic barley root tissue.

Fraction	Enzyme Activity	Specific Activity	Purification	Yield
	units	*units/mg protein*	*-fold*	*%*
Crude	1890	6	1	100
35% Supernat.[a]	1334	14	2	71
65% Pellet[a]	1329	19	3	70
Gel Filtration	1094	85	13	58
Q-Sepharose	578	266	42	27
Mono-P	53	699	109	3
Mono-Q	59	2231	349	3

[a] The 35% $(NH_4)_2SO_4$ supernatant and 65% $(NH_4)_2SO_4$ pellet are as described in the text.

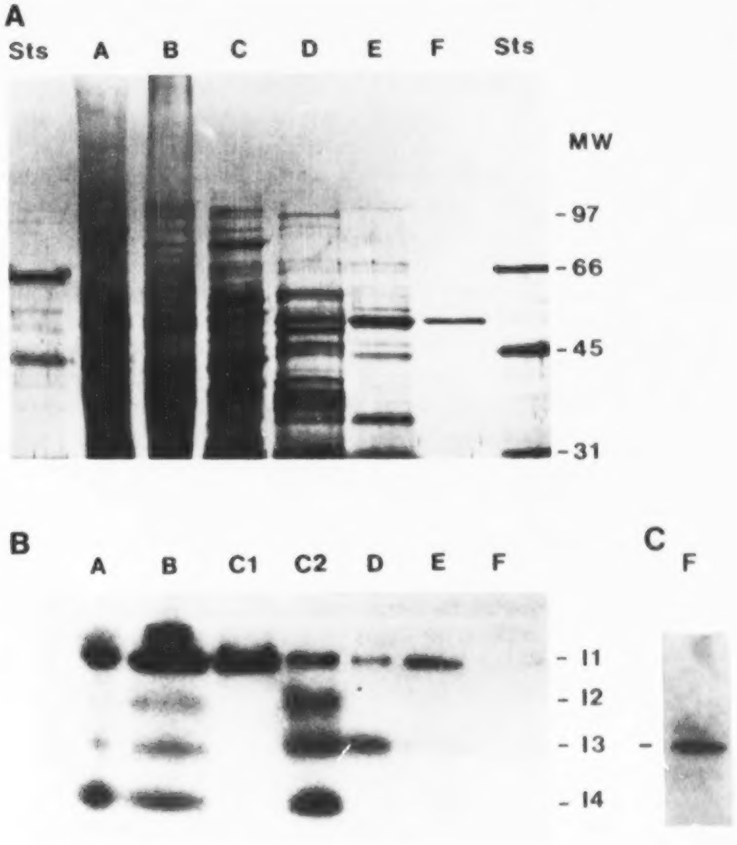

Figure 2.2. Progress of the AlaAT purification as determined by native and SDS-PAGE. A, Aliquots from each of the purification steps were subjected to SDS-PAGE on 7% (w/v) gels and visualized by silver staining. The amount of protein loaded in each lane is indicated in brackets. Lane A: clarified homogenate (12μg); Lane B: 35 to 65% (saturation) $(NH_4)_2SO_4$ resuspended pellet (20μg); Lane C: gel filtration Peak 2 pooled fractions (12μg); Lane D: Q-Sepharose active fractions (9μg); Lane E: Mono-P active fractions (8μg); Lane F: Mono-Q active fractions (1.2μg); Sts: molecular mass standards. B, Aliqots from the active fractions of each purification step were subjected to native-PAGE on 7% (w/v) gels and AlaAT activity visualized as described in the text. Fractions are the same as above with the following exceptions. Lane C1: gel fitration Peak 1 pooled fractions; C2 gel filtration Peak 2 pooled fractions. The amount of protein loaded in each lane is indicated below. (A,60μg; B,105μg; C1,20μg; C2,20μg; D,9μg; E,8μg; F,1.2μg). C, silver stain of lane F from panel B. I1 to I4 refer to the different isoforms present.

polypeptide band of 50 kD when run on SDS-PAGE and stained with either Coomassie blue or silver stain (Fig. 2.2A, lane F). The native molecular mass of the enzyme as estimated by gel filtration of the final preparation was found to be approximately 97 kD. This suggests that AlaAT is a dimer.

Figure 2.2B illustrates a native gel which was stained for AlaAT activity. In the clarified homogenate (lane A) and the 35 to 65% saturation $(NH_4)_2SO_4$ resuspended pellet (lane B), there were a number of bands showing AlaAT activity. However, peak 1 of the gel filtration column (Fig. 2.1) contained a single intense activity band of low mobility (Lane C1, Isoform 1; see Addendum, Section 2.6). This isoform was present until the final step of purification, when the only activity band was isoform 3, which was very faint in the final two steps. When lane F was silver stained to detect total protein, a single band was present which comigrated with the isoform 3 activity band (Fig. 2.2C, lane F). Isoforms 2 and 4 are visible in Figure 2.2B, lanes A,B, and C2 but could not be detected after Q-Sepharose ion exchange chromatography. Presumably these two isoforms either eluted differently on Q-Sepharose but were undetectable due to their relatively low activity, or they co-eluted with isoform 3 in an inactive form.

2.3.3 *Effect of PLP on AlaAT Activity*

A number of different researchers have found that pyridoxyl-5-phosphate (PLP) increases aminotransferase activity or binding properties to chromatography columns (Hatch 1973, Hondred et al. 1985). We incubated each pooled fraction containing AlaAT activity with 10 mM PLP, however the presence of PLP had no positive or negative effect on enzyme activity at any stage of purification. Moreover, the presence of PLP in the gel filtration and ion exchange steps had no effect on the binding characteristics of this enzyme to the respective columns.

2.3.4 Characterization of AlaAT

The purified AlaAT enzyme has K_m values of 17 mM and 5 mM for alanine and 2-oxoglutarate, and K_m values of 0.06 mM and 1.0 mM for pyruvate and glutamate, respectively. The pH optimum of the enzyme was fairly broad, with the optimum being between 8.0 and 8.3 in the alanine → pyruvate direction. The purified AlaAT shows a high specificity for alanine. It cannot use serine or aspartate as an amino donor. In addition, AlaAT can utilize 2-oxoglutarate as a keto donor but not glyoxylate. Table 2.2. illustrates the effect of the different inhibitors that were tested on enzyme activity. Of the different inhibitors that were tested, amino-oxyacetate was by far the most effective inhibitor.

2.4 Discussion

In previous investigations of aminotransferase activity in specific plant tissues, a number of different isoforms have been found that can be separated by either gel filtration or ion exchange chromatography. For example, Hatch (1973) identified two different aspartate aminotransferases (AAT) and three alanine aminotransferases in leaves of *Atriplex spongiosa* using ion exchange

Table 2.2: Effect of various inhibitors of the activity of the hypoxically induced AlaAT.

Enzyme activity in the presence of inhibitors is expressed relative to the control set at 100%.

Compound	Concentration Tested			
	10 μM	1 mM	10 mM	100 mM
		%		
Amino-oxyacetate	5	2	1	1
α-aminobutyrate	ND[a]	76	74	66
Glutarate	ND	64	6	1
Semicarbazide	ND	60	3	1

[a] ND = Not determined.

chromatography. Numazawa et al. (1989) identified two isoforms of AAT in leaf tissue of *Panicum maximum*, whereas De-Eknamkul and Ellis (1987) identified four isoforms of tyrosine aminotransferase in cell cultures of *Anchusa officinalis* using ion exchange chromatography. Similarly, in barley root tissue we have found two different AlaAT isoforms using column chromatography and four isoforms by gel electrophoresis.

Our conclusion that the second peak of AlaAT activity obtained following gel filtration chromatography is the hypoxically induced isoform is based on three observations. First, peak 2 is the major peak of activity representing 87% of the total activity in crude extracts prepared from hypoxically induced roots (Fig. 2.1). Because AlaAT activity increases approximately 4-fold after 4 d of hypoxic induction (Good and Crosby 1989b), at least 80% of the AlaAT activity should be of the hypoxically induced form. Therefore, the major peak of activity should correspond to the hypoxically induced isoform. Second, the Superdex G-200 elution profile for AlaAT activity from aerobic versus hypoxic root tissue clearly demonstrates that peak 2 is the hypoxically induced isoform (Fig. 2.1). Third, we have also partially purified AlaAT initially using an ion exchange column first, followed by gel filtration and found that the same peak is hypoxically induced (data not shown).

Native PAGE (Fig. 2.2B) shows that four AlaAT isoforms exist in the crude preparation. Although isoform 1 yielded the most intense activity band of the four, we have found that the intensity of the band on a gel does not directly reflect the level of activity in the sample prior to running the gel. For example, in Figure 2.2B, the activity loaded onto lane D (the Q-Sepharose fraction) was four times that of lane C1 (peak 1 from gel filtration), even though the single band in C1 shows substantially higher intensity than the bands in lane D. In addition, the homogeneous AlaAT sample possesses very little activity on nondenaturing gels (Fig. 2.2B, lane F), however when stained for total protein, a band is visible which comigrates with isoform 3 (Fig. 2.2C, lane F). Therefore isoform 3 likely loses much of its activity when run on a native gel, either in a crude or a purified sample. This finding may explain why Good and Crosby (1989b) and Watson et al. (1992) found no visible increase in any of the AlaAT bands of hypoxically induced root samples.

The purified native AlaAT had an estimated molecular mass of 97 kD compared to subunit molecular mass of 50 kD indicating that this enzyme is probably a homodimer. Purified AlaATs from broomcorn millet (*Panicum miliaceum*) leaves and from *Chlamydomonas* are likely dimers as well, with subunits sizes of 50 and 45 kDa, respectively (Son et al. 1991, Lain-Guelbenzu et al. 1991). Other plant aminotransferases have been reported to have native molecular masses ranging from 80 to 220 kD and are either dimers or tetramers with subunits ranging from 40 to 56 kD (De-Eknamkul and Ellis 1987, Hondred et al. 1985, Numazawa et al. 1989, Wightman and Forest 1978).

Aminotransferases in plants have been shown to exhibit broad substrate specificities (Hatch 1973, Hondred et al. 1985, Numazawa et al. 1989). The recent purification of AlaAT from *Chlamydomonas* and *P. miliaceum* showed that the purified enzyme is quite specific in terms useable substrates (Lain-Guelbenzu et al.

1991, Son et al. 1991). The ability of the *Chlamydomonas* AlaAT to use glyoxylate in addition to 2-oxoglutarate as a keto donor is an exception to this specificity. Our purified AlaAT was very specific in its amino and keto acid substrates, providing more evidence that, unlike other plant aminotransferases, plant AlaATs are quite specific in the substrates that they utilize. A second distinctive feature of this enzyme is that PLP does not have any effect on either enzyme activity or column binding. Hondred et al. (1985) were able to purify a serine:glyoxylate aminotransferase because this enzyme binds to DEAE cellulose in the presence, but not in the absence of PLP. However unlike serine:glyoxylate aminotransferase, the hypoxically induced barley root isoform of AlaAT binds to Q-Sepharose irrespective of whether PLP is present. These data suggest that the hypoxically inducible AlaAT of barley roots binds PLP very strongly, as the addition of exogenous PLP does not enhance its stability.

The sub-cellular location of the hypoxically inducible AlaAT has not been determined, as we were unsuccessful in isolating intact organelles from barley roots. AlaATs from other species and tissues have been localized to the cytosol, microbodies and mitochondria (Hatch et al. 1987, Biekman and Feierabend 1982, Noguchi and Hayasha 1981). It is, therefore, difficult to speculate on the sub-cellular location of barley root AlaAT.

A number of different compounds were tested for their ability to inhibit this specific isoform. The purified AlaAT was strongly inhibited by amino-oxyacetate, but only weakly inhibited by a number of other compounds that are known to inhibit beef heart alanine aminotransferase (Bulos and Handler 1965). Lain-Guelbenzu et al. (1991) showed that amino-oxyacetate was also a very strong inhibitor of AlaAT activity in *Chlamydomonas*.

Hypoxia or anoxia in maize roots results in the synthesis of a number of anaerobic proteins which have been detected on two-dimensional gels (Sachs et al. 1980). Several of these anaerobic proteins have been identified including ADH, pyruvate decarboxylase, aldolase and glucose phosphate isomerase. We have identified and purified another of the anaerobically induced proteins, an isoform of AlaAT from barley root tissue.

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2.6 Addendum

Subsequent to the publication of the data presented in this chapter, it was brought to our attention that the AlaAT activity stain of nondenaturing gels may also stain for glutamate dehydrogenase (GDH; MM Sachs, personal communication, Watson et al. 1992). In this assay, the AlaAT reaction is coupled to NADH oxidation by LDH, as described in Section 2.2.6 and diagrammed in Fig. 2.3A. The commercial preparation of LDH used in this reaction is suspended in ammonium sulphate. Since ammonium ion and 2-oxoglutarate rather than alanine and 2-oxoglutarate are consumed in GDH reactions, this AlaAT stain could inadvertantly detect GDH activity, as ammonoum ions present in the LDH solution would be a substrate for GDH (Fig. 2.3B).

In order to determine if any of the four bands seen in Fig. 2.2B were in fact GDH, several replicate samples were run on non-denaturing gels and stained using various substrates. Each of the gel strips contained protein from peak 1 and peak 2 fractions of the gel filtration effluent (Fig. 2.4). Fig. 2.4A shows the single band of activity which is present in the gel filtration peak 1 sample, and the four bands present in the gel filtration peak 2 sample, similar to that of lanes C1 and C2 in Fig. 2.2B. This gel was stained the same as that shown in Fig. 2.2B, and contained alanine, 2-oxoglutarate, NADH and LDH suspended in ammonium sulphate. When a gel strip is stained as in Fig. 2.3A, but without adding alanine as a substrate, only isoform 1 is present (Fig. 2.4B). Since alanine was not added to this reaction stain,

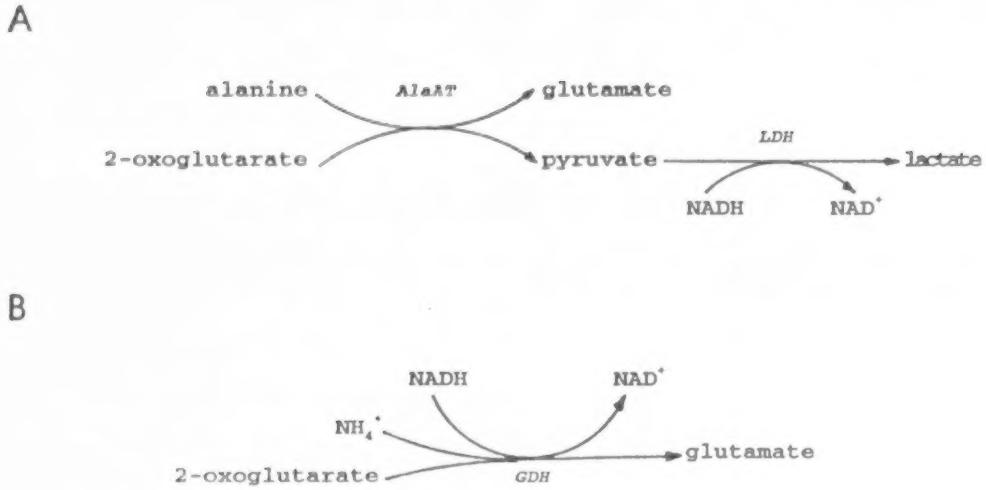

Fig. 2.3. Diagrammatic representation of the A) AlaAT and B) GDH reactions used to stain their for the corresponding activities on nondenaturing gels. Both stains are buffered with 50 mM Tris-HCl (pH 8.0). The coupled AlaAT stain contains alanine, 2-oxoglutarate, NADH and LDH. The GDH stain contains 2-oxoglutarate, ammonium ions, and NADH. The activity bands are visible by the non-fluorescence of NAD^+ on a background of fluorescent NADH.

Alanine	+			+	+
2-oxoglutarate	+	+	+	+	+
LDH-ammonium	+	+			
LDH-crystalline				+	+
NH_4Cl			+		
Amino-oxyacetate					+
	A	B	C	D	E

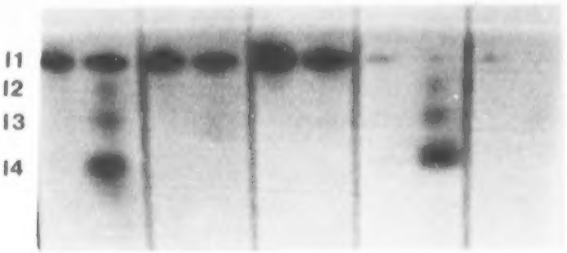

Fig. 2.4. Nondenaturing gel strips (A to E) each containing protein from peak 1 and peak 2 fractions of the gel filtration eluate, similar to that run in lanes C1 and C2 of Fig. 2.2B. Strips were stained using different substrates as indicated above each strip (+). In addition, all stains contained 50 mM Tris-HCl (pH 8.0) and 2 mM NADH in a 50 mL volume. The concentrations of the other substrates are: 30 mM L-alanine; 10 mM 2-oxoglutarate; 20 units/mL LDH (either resuspended in ammonium sulphate or in a crystalline form); 20 mM NH_4Cl; and 1 mM amino-oxyacetate. Gel strip E was preincubated in amino-oxyacetate for 30 min to allow for its penetration into the gel. The gels were run under similar conditions as described in Section 2.2.6. Twenty μg protein was loaded into each lane.

AlaAT activity bands would not be present. Therefore, isoform 1 would appear to have GDH activity. To further prove that isoform 1 is GDH, a stain specific for GDH was used which lacked alanine and LDH, but contained ammonium chloride, 2-oxoglutarate and NADH. This stain only identified isoform 1 as being GDH (Fig. 2.4C).

A stain specific for AlaAT activity was used which contained a crystalline LDH preparation containing no ammonium ions (Sigma L1254), in place of the liquid LDH preparation containing ammonium ions. Here, isoforms 2, 3 and 4 stain intensely on the gel strip (Fig. 2.4D). The faint staining of isoform 1 (GDH) is likely due to the residual ammonium present from the ammonium persulphate used to polymerize the gel. Fig. 2.4E shows a gel strip stained specifically for AlaAT as just described, however the gel was preincubated in the presence of amino-oxyacetate, a known inhibitor of AlaAT activity (Hopper and Segal 1964). Here, the isoforms 2,3 and 4 disappear, further proving that these bands have AlaAT activity.

In conclusion, this data indicates that isoform 1 in Fig. 2.2B is in fact GDH. Since only this band is present in the sample from peak 1 of the gel filtration eluate (Fig. 2.2B lane C1), then peak 1 appears to contain only GDH activity. Watson et al. (1992) used similar controls as performed here and found that maize root samples stained for AlaAT also stained for GDH. The GDH band in maize was also the slowest of the bands present. As a result, only three isoforms of AlaAT activity are present in barley roots. Isoform 2 is thus defined as the hypoxically inducible isoform from this point on in the thesis, rather than isoform 3 as described in this chapter.

Since the spectrophotometric assay used in monitoring the activity of AlaAT throughout the purification process also takes advantage of this coupled assay (See Section 2.2.3), it was expected that GDH activity would be inadvertantly measured here as well. The substitution of crystalline LDH for suspended LDH in the spectrophotometric assay buffer was performed, as this buffer would only assay for AlaAT activity. When the eluting fractions of the gel filtration column were assayed using this buffer, peak 1 again was lost providing evidence that peak 1 is GDH. In the assay of crude preparations, approximately 10% of the activity in hypoxically treated samples was lost using this assay buffer compared to the original assay buffer, indicating that GDH activity is 10% that of AlaAT. GDH activity is not hypoxically inducible in root samples.

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3 Hypoxically inducible barley alanine aminotransferase: molecular cloning and expression analysis

3.1 Introduction

The identification and purification of the hypoxically inducible AlaAT described in the previous chapter permits a further characterization of the expression patterns of this enzyme. Although spectrophotometric analysis shows the induction of AlaAT-2 activity under hypoxic stress, this does not tell us at what step during gene expression induction occurs. Other ANPs such as ADH and LDH show a correlation between the increase in activity and protein concentration during hypoxic stress. The increase in ADH protein has been shown to result from both an increase in transcription of the *Adh* gene and stability of the Adh mRNA (Rowland and Strommer 1986). Since AlaAT activity increases similarly to that of other ANPs, the types of regulation of AlaAT expression may also be similar.

In addition to being anaerobically inducible, AlaAT activity increases in leaves of broomcorn millet (*Panicum miliaceum*) upon exposure to light (Son et al. 1991). In *P. miliaceum*, AlaAT functions in the aspartate/alanine shuttle in the NAD-malic enzyme type C_4 cycle of photosynthesis that this species employs. A cDNA

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encoding this light inducible AlaAT has recently been isolated and sequenced (Son and Sugiyama 1992).

This chapter describes the molecular cloning of a hypoxically induced AlaAT cDNA from barley roots, and the characterization of AlaAT expression at the level of the mRNA and protein. Evidence is also presented which indicates that AlaAT-2 expression of *P. miliaceum* is inducible by hypoxia as well as by light, a condition which is unique among ANPs studied to date. In addition, the isolation and partial sequence analysis of an AlaAT genomic clone from maize is described. Since the isolation of a genomic clone will eventually allow for the analysis of the upstream regulatory elements, our choice of isolating this clone from maize rather than barley will allow a more direct comparison to the regulatory regions of maize ANP genes already studied by other researchers.

3.2 Materials and Methods

3.2.1 Plant culture

Barley (*Hordeum vulgare* L. cv. Leduc) seedlings were germinated, grown hydroponically, and hypoxically treated as described in Section 2.2.1. Broomcorn millet (*Panicum miliaceum*) seeds were germinated on moist filter paper in the dark at room temperature for 5 d, and transferred to hydroponic tanks as above.

3.2.2 Extraction and assay of AlaAT

Proteins were extracted by grinding root or leaf tissue in a mortar and pestle in extraction buffer (0.1 M Tris-HCl, pH 8.0, 10 mM DTT and 10% (v/v) glycerol). AlaAT activity was assayed spectrophotometrically essentially as described in Section 2.2.3., differing only in that crystalline LDH preparation (Sigma L1254) replaced the ammonium sulphate suspended LDH preparation in the assay.

3.2.3 Preparation of antiserum against AlaAT

The hypoxically inducible AlaAT-2 was purified from 4-day hypoxically induced barley root tissue as described previously (Section 2.2.2), but eliminating the Mono P (Pharmacia) chromatofocusing step in order to increase the yield of protein. Fifty µg of native AlaAT protein mixed in Freund's complete adjuvant was injected both subscapularly and intramuscularly into Flemish giant lop-eared cross rabbits, followed by booster injections of 30 µg protein mixed in Freund's incomplete adjuvant 21 and 49 d after the initial injection. A preimmune sample was taken prior to the initial injections. Serum was harvested 3 weeks after the final injection of protein. Polyclonal antiserum raised against the light inducible isoform of *P. miliaceum* AlaAT-2 was a gift from Deyoung Son (Nagoya University, Japan).

3.2.4 Immunotitration of AlaAT

The polyclonal antibody was tested for specificity by immunotitration assays of crude root extracts. Varying amounts of serum was diluted in extraction buffer to a volume of 50 µL, and mixed with 200 µL of cleared, crude root extract (0.1 gm tissue/mL extraction buffer) and incubated at room temperature for 1 h and then overnight at 4°C. The sample was then centrifuged for 15 min at 13,000 rpm in a benchtop microfuge at 4°C, and the supernatant assayed spectrophotometrically for AlaAT activity.

3.2.5 Gel electrophoresis and blotting of proteins to nitrocellulose

Non-denaturing and SDS-PAGE was performed as described previously (Section 2.2.6). Staining of non-denaturing gels was also as described in Section 2.2.6, differing only in that crystalline LDH (Sigma, L1254) was added to the stain rather than LDH resuspended in ammonium sulphate. Electroblotting of both gel types to nitrocellulose was performed in 5 mM Tris, 38 mM glycine (pH 8.3) at 4°C overnight according to the manufacturers specifications (Trans-blot transfer cell, BioRad). Antibody staining was similar to that of Good and Crosby (1989a). The nitrocellulose was incubated in TBST (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween 20) containing 4% no-fat milk powder at 4°C overnight. The nitrocellulose was then incubated in the above solution containing AlaAT primary antibody (1:10,000 dilution) at room temperature for two h, then washed three times for 3 min each in the TBST/milk powder solution. The blots were then incubated in TBST/milk powder containing a 1:7500 dilution of goat anti-rabbit IgGs linked to alkaline phosphatase (Promega) for 1 h and then washed as above. The blots were then again washed as described above, and then stained in alkaline phosphatase buffer (10 mM Tris-HCl [pH 9.5], 100 mM NaCl, 5 mM $MgCl_2$) containing 0.3 mg/mL nitroblue tetrazolium and 0.2 mg/mL of 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

3.2.6 Determination of amino acid sequence

AlaAT protein was cleaved using the endoproteinase Lys C (Boehringer Mannheim), the peptides separated on a C_{18} reverse phase HPLC column, and two of the liberated peptides were amino-terminal sequenced. The cleavage and sequencing was performed by William Lane of the Harvard Microchemistry Facility. Twenty and 32 residues of sequence were obtained from the two peptides, respectively (Fig. 3.2).

3.2.7 DNA manipulations

All standard DNA manipulations such as colony and plaque growth and DNA preparations and Southern blotting were carried out according to Sambrook et al. (1989).

3.2.8 Polymerase chain reaction (PCR)

Degenerate oligonucleotide primers were synthesized based on the 32 amino acid peptide sequence described in Section 3.2.6. The 5' primer was 64-fold degenerate based on the amino acid sequence EHAFNK, and the 3' primer was 96-

fold degenerate based on the amino acid sequence FPQICL (Fig. 3.2). A 6-bp sequence (CAAAGCTT) was present 5' to the 64-fold degenerate primer, and a 7-bp sequence (GGAATTC) was added 5' to the 96-fold degenerate primer. These sequences contain an EcoR I and Hind III restriction enzyme site, respectively, and were included to facilitate subcloning the PCR product. A cDNA library (gift from Andrew Hanson) was constructed from poly-(A)$^+$ mRNA isolated from 2 d hypoxically induced barley root tissue (cv. Himalaya) by vector priming as described previously (Alexander et al. 1984). Plasmid DNA from the cDNA library was extracted and used as the template for PCR. Reaction mixtures contained 10 ng of template DNA, 100 pmol of each oligonucleotide pool, 20 mM Tris-HCl pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 4 mM Mg_2SO_4, 0.1% Triton X-100, 100 µg/ml BSA, and 1 unit of Vent polymerase (New England Biolabs) in a volume of 50 µL. The first three thermal cycles consisted of 94°C for 0.5 min, 37°C for 1 min, and 72°C for 1 min. The next 30 cycles consisted of 94°C for 0.5 min, 50°C for 0.5 min, and 72°C for 0.5 min. The PCR product (93 base pairs, see Figs. 3.1 and 3.2) was gel purified, sequenced to confirm a match with the 32 amino acid peptide sequence, and then used a probe for library screening. The double stranded sequencing reactions were as described in Section 3.2.11, with the 5' and 3' degenerate oligonucleotides described above used to prime the sequencing reaction. The PCR generated probe was labelled with ^{32}P-dCTP using a standard random priming protocol (Sambrook et al. 1989), however, the degenerate 5' and 3' oligonucleotides described above were used in place of random primers in the labelling reaction.

3.2.9 Screening of the cDNA library

The cDNA library, generated from 2-day hypoxically induced barley roots described above, was plated at a density of 10,000 colonies per plate on 10-150 mm LB plates containing 50 mg/L ampicillin. Colonies were lifted onto nitrocellulose membranes (Nitroplus, MSI). Colony hybridizations were performed at 65°C for 12 h using the 93 bp PCR generated probe, in 40 mL of hybridization buffer (5X SSC, 5X Denhardt's solution, 1% (w/v) SDS and 100 µg/mL sonicated salmon sperm DNA). Blots were washed twice in 2X SSC for 5 min at room temperature, and twice in 2X SSC and 0.1% SDS for 15 min at 65°C. Putative positive clones were amplified in liquid culture, and the DNA extracted. Sma I digests were then used to excise the cDNA inserts from the vector and the DNA analyzed by Southern analysis using the PCR product as a probe.

3.2.10 Screening of the genomic library

A genomic library of leaf genomic DNA from maize (cv W22) prepared in our laboratory was cloned into λ-DASH II by Stratagene Corp.. Approximately 500,000 plaques were plated on 15-150 mm plates, and were lifted onto nitrocellulose membranes (Nitro-plus MSI). A probe was made by labelling the AlaAT cDNA clone (pCAlaAT-3a, See Results) with ^{32}P-dCTP using a standard random priming protocol (Sambrook et al. 1989). Plaque hybridizations were performed at 60°C for 14 h in 30 mL hybridization buffer. Blots were washed twice in 2X SSC for 5 min at room temperature, and twice in 2X SSC and 0.1% SDS for 15 min at 60°C.

Putative positive plaques were then isolated and replated in a secondary and a tertiary screen. DNA was extracted from the positive clone and a restriction map was generated.

3.2.11 Sequence analysis

Genomic and cDNAs were subcloned into pT7T319U (Pharmacia) and nested deletions were made into the insert from both directions using an exo III/mung bean deletion kit (Stratagene). Double stranded sequencing of both DNA strands using T3 and T7 primers was performed by the dideoxy chain termination method (Sanger et al. 1977) using a sequencing kit (Sequenase, United States Biochemicals).

3.2.12 Preparation of total RNA

After aerobic or anaerobic treatment, 2 gm of root tissue from 2-3 week old barley seedlings was washed, blotted, quickly frozen in liquid nitrogen, and total RNA isolated by a method similar to that described by Yeh et al. (1990). Frozen tissue was homogenized to a fine powder and mixed with 2 volumes (w/v) of guanidinium buffer (7.5 M guanidinium-HCl, 25 mM sodium citrate, 0.5% (w/v) lauryl sarcosinate and 0.1 M 2-mercaptoethanol). The mixture was centrifuged at 4°C for 10 min at 10,000 X g, and the supernatant filtered through sterile miracloth. Protein was then extracted twice with 1 volume of phenol/chloroform/isoamyl alcohol (24/24/1), and once with 1 volume of chloroform/isoamyl alcohol (24/1). The

RNA was precipitated by the addition of an equal volume of isopropanol and 1/10 volume of 3 M sodium acetate (pH 5.2) and incubated at -20°C overnight. The RNA was pelleted in a microfuge at 13,000 rpm at 4°C for 15 min, and the pellet resuspended in sterile TE (10 mM Tris-HCl [pH8.0], 1mM EDTA [pH 8.0]).

3.2.13 RNA gel and slot blot analysis

For Northern blots, 10-15 μg RNA was denatured in denaturation buffer (2 μL 10X MOPS buffer [0.2 M morpholinopropanesulfonic acid, 50 mM sodium acetate, 10 mM EDTA and the pH brought to 7.0 with sodium hydroxide], 3.5 μL 37% formaldehyde and 10μL deionized formamide to a total volume of 20 μL). The RNA was then electrophoresed through 1.2% formaldehyde agarose gels (6% formaldehyde, 1X MOPS buffer) at 5 V/cm for 4-5 h and then transferred to nylon membranes (Gene Screen Plus, Dupont) by the capillary method (Selden 1989). For slot blots, 3-4 μg of denatured RNA was applied into each slot of the slot blotter (Schleicher and Schuell, Minifold II) followed by washing of each slot with 400 μL of 10X SSC. Hybridization of Northern and slot blots was in 50% formamide, 5X SSC, 1% SDS, 5X Denhardt's solution, 100 μg/mL sonicated salmon sperm DNA and 10% polyethylene glycol. Blots were hybridized at 42°C for 12 h, and washed twice in 2X SSPE at room temperature for 15 min each, twice in 2X SSPE at 65°C for 45 min each, and twice in 0.1X SSPE at room temperature for 15 min each. Blots were exposed to X-ray film for 2-12 h at -80°C. AlaAT slot blot data were normalized for loading differences by probing replicates with a soybean 18S rDNA probe. Linearity of band intensity on the film was determined by loading dilutions of a particular sample prior to probing.

3.2.14 Densitometric analysis

RNA slot blots and protein gel immunoblots were densitometrically scanned using the BioPhonics Gel Print imaging system and the Gel Print Tool Box (Version 2.0) computer program.

3.3 Results

3.3.1 Isolation of a cDNA corresponding to the hypoxically induced AlaAT

The PCR reaction using degenerate primers and a cDNA library template (described in Section 3.2.8) produced the expected 93 bp product (Fig. 3.1). This product was gel purified and sequenced to confirm its identity to the corresponding amino acid sequence shown in Fig. 3.2. It was then radioactively labelled and used to screen a hypoxically induced barley root cDNA library. Two positive clones were identified, each containing inserts of about 550 bp in length. One of these inserts was used to reprobe the cDNA library. From this screen, another 7 positive clones were identified, most of which contained inserts 500-700 bp in length, however, one insert was 1.75 kb in length. This insert was subcloned and designated as pCAlaAT-3a. The nucleotide sequence of pCAlaAT-3a (Fig. 3.2) indicates that this cDNA has an open reading frame of 1446 bp (482 amino acids, protein molecular mass of

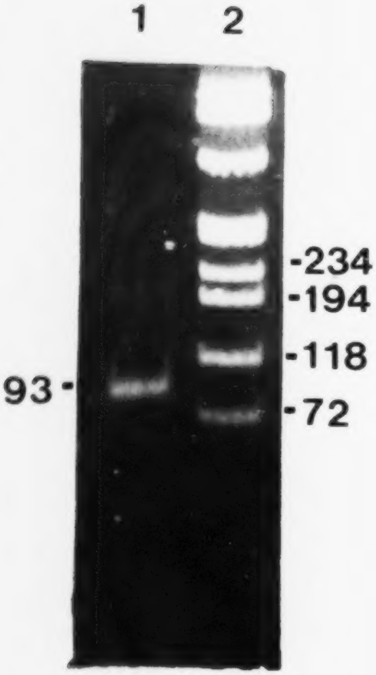

Figure 3.1 A 12.5% acrylamide gel showing the 93 base pair product (lane 1) of the PCR reaction generated by using the cDNA library as a template and degenerate oligonucleotides as primers. Lane 2, ϕX174 Hae III standards.

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GGCCACAAAACCGCGGAAAGAGATAGACGGACAGCTAGAGGCGTCGGAAGATACTCGCTGCTCTGCCGCCCCCTTCGTCTTAGTTGATCTCGCC      94

ATGGCTGCCACCGTCGCCGTGGACAACCTGAACCCCAAGGTTTTAAAATGTGAGTATGCTGTGCGTGGAGAGATTGTCATCCATGCTCAGCGCTTG    190
M  A  A  T  V  A  V  D  N  L  N  P  K  V  L  K  C  E  Y  A  V  R  G  E  I  V  I  H  A  Q  R  L       32

CAGGAACAGCTAAAGACTCAACCAGGGTCTCTACCTTTTGATGAGATCCTCTATTGTAACATTGGGAACCCACAATCTCTTGGTCAGCAACCAGTT    286
Q  E  Q  L  K  T  Q  P  G  S  L  P  F  D  E  I  L  Y  C  N  I  G  N  P  Q  S  L  G  Q  Q  P  V       64

ACATTCTTCAGGGAGGTTCTTGCCCTTTGTGATCATCCAGACCTGTTGCAAAGAGAGGAAATCAAAACATTGTTCAGTGCTGATTCTATTTCTCGA    382
T  F  F  R  E  V  L  A  L  C  D  H  P  D  L  L  Q  R  E  E  I  K  T  L  F  S  A  D  S  I  S  R       96

GCAAAGCAGATTCTTGCCATGATACCTGGAAGAGCAACAGGAGCATACAGCCATAGCCAGGGTATTAAAGGACTTCGTGATGCAATTGCTTCTGGG    478
A  K  Q  I  L  A  M  I  P  G  R  A  T  G  A  Y  S  H  S  Q  G  I  K  G  L  R  D  A  I  A  S  G      128

ATCGCTTCACGAGATGGATTCCCTGCTAATGCTGATGACATTTTTCTCACAGATGGAGCAAGTCCTGGGGTGCACCTGATGATGCAATTACTGATA    574
I  A  S  R  D  G  F  P  A  N  A  D  D  I  F  L  T  D  G  A  S  P  G  V  H  L  M  M  Q  L  L  I      160

AGGAATGAGAAAGATGGCATTCTTGTCCCGATTCCTCAGTACCCCTTGTACTCGGCTTCCATAGCTCTTCATGGCGGAGCTCTTGTCCCATACTAT    670
R  N  E  K  D  G  I  L  V  P  I  P  Q  Y  P  L  Y  S  A  S  I  A  L  H  G  G  A  L  V  P  Y  Y      192

CTCAATGAATCGACGGGCTGGGGTTTGGAAACCTCTGATGTTAAGAAGCAACTTGAAGATGCTCGGTCAAGAGGCATCAACGTTAGGGCTTTGGTG    766
L  N  E  S  T  G  W  G  L  E  T  S  D  V  K  K  Q  L  E  D  A  R  S  R  G  I  N  V  R  A  L  V      224

GTTATCAATCCAGGAAATCCAACTGGACAGGTACTTGCTGAAGAAAACCAATATGACATAGTGAAGTTCTGCAAAAATGAGGGTCTTGTTCTTCTA    862
V  I  N  P  G  N  P  T  G  Q  V  L  A  E  E  N  Q  Y  D  I  V  K  F  C  K  N  E  G  L  V  L  L      256

GCTGATGAGGTATACCAAGAGAACATCTATGTTGACAACAAGAAATTCCACTCTTTCAAGAAGATAGTGAGATCCTTGGGATACGGCGAGGAGGAT    958
A  D  E  V  Y  Q  E  N  I  Y  V  D  N  K  K  F  H  S  F  K  K  I  V  R  S  L  G  Y  G  E  E  D      288

CTCCCTCTAGTATCATATCAATCTGTTTCTAAGGGATATTATGGTGAGTGTGGTAAAAGAGGTGGTTACTTTGAGATTACTGGCTTCAGTGCTCCA   1054
L  P  L  V  S  Y  Q  S  V  S  K  G  Y  Y  G  E  C  G  K  R  G  G  Y  F  E  I  T  G  F  S  A  P      320

GTAAGAGAGCAGATCT[illegible]AAAATAGCATCAGTGAACCTATGCTCCAATATCACTGGCCAGATCCTTGCTAGTCTTGTCATGAACCCACCAAAGGCT   1150
V  R  E  Q  I  Y  K  I  A  S  V  N  L  C  S  N  I  T  G  Q  I  L  A  S  L  V  M  N  P  P  K  A      352

AGTGATGAATCATACGCTTCATACAAGGCAGAAAAAGATGGAATCCTCGCATCTTTAGCTCGTCGTGCGAAGGCATTGGAGCATGCATTCAATAAA   1246
S  D  E  S  Y  A  S  Y  K  A  E  K  D  G  I  L  A  S  L  A  R  R  A  K  A  L  E  H  A  F  N  K      384

CTTGAGGGAATTACTTGCAACGAGGCTGAAGGAGCAATGTACGTGTTCCCTCAAATCTGTCTGCCACAGAAGGCAATTGAGGCTGCTAAAGCTGCT   1342
L  E  G  I  T  C  N  E  A  E  G  A  M  Y  V  F  P  Q  I  C  L  P  Q  K  A  I  E  A  A  K  A  A      416

AACAAAGCACCTGATGCATTCTATGCTCTTCGTCTCCTCGAGTCGACTGGAATCGTCGTTGTCCCTGGATCAGGATTTGGCCAGGTTCCTGGCACA   1438
N  K  A  P  D  A  F  Y  A  L  R  L  L  E  S  T  G  I  V  V  V  P  G  S  G  F  G  Q  V  P  G  T      448

TGGCACTTCAGGTGCACGATCCTTCCGCAGGAGGATAAGATCCCGGCAGTCATCTCCCGCTTCACGGTGTTCCATGAGGCGTTCATGTCAGAGTAT   1534
W  H  F  R  C  T  I  L  P  Q  E  D  K  I  P  A  V  I  S  R  F  T  V  F  H  E  A  F  M  S  E  Y      480

CGTGACTAAACTGGTGCAACATGTGGGATTACATACAACCCTCATGGGGTTTTCGTAGGCGTTCTTGGTTTTGCCCCCCCCCCCCTTCTCTCTCTC   1630
R  D                                                                                                 482

TCTCTCTCTGACAGCATCCTCCTCTAGATGAGACAAAATAAAGCAAAGCCATGTCATCCTTAAAAAAAAAA     1701
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Figure 3.2. Nucleotide and deduced amino acid sequence of a cDNA encoding AlaAT-2 from barley. The putative polyadenylation signal is indicated by the dashed line. Amino acid sequence of the amino termini of two peptides produced by LysC endoproteinase digestion of AlaAT-2 protein is underlined. The sequences corresponding to the oligodeoxynucleotide pools used to prime the PCR reaction are found below the arrows.

52,885 Da), a 5' untranslated region of 94 bp, and a 3' untranslated region of 151 bp. Fifty A residues were present at the end of the cDNA, and a polyadenylation signal consensus sequence was present 20 bp from the 3' terminus (Fig. 3.2). The remaining positive cDNA clones all hybridized to the 3' untranslated region of pCAlaAT-3a and are assumed to be truncated versions of the same cDNA. Figure 3.3 compares the deduced amino acid sequence of pCAlaAT-3a to that of the light inducible AlaAT from *P. miliaceum*. These two sequences have 88% nucleotide identity within the coding region and 90% deduced amino acid identity. Previously identified amino acids crucial for pyridoxal phosphate (PLP) binding in *P. miliacuem* AlaAT (Son and Sugiyama 1992) are conserved in barley AlaAT, including a lysine residue (position 299) which is presumed to be important in providing the aldimide bond necessary for binding PLP (Son and Sugiyama 1992). Although sequence differences are found between these two AlaATs, there are long stretches of identical sequence present (e.g., residues 38-77, 313-351, 408-450, Fig. 3.3). Twenty-nine amino acids conserved between human, rat and *P. miliaceum* AlaATs and *P. miliaceum* aspartate aminotransferases (Son and Sugiyama 1992), are also conserved in barley AlaAT (data not shown).

3.3.2 Hypoxically induced accumulation of AlaAT mRNA

The concentration of AlaAT mRNA present in aerobic and hypoxic barley root tissue was estimated by RNA gel and slot blotting (Fig. 3.4). A single band of approximately 1.75 kb was visible when RNA gel blots were probed with pCAlaAT-

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                                                                                        90
MAATVAVDNLNPKVLKCEYAVRGEIVIHAQRLQEQLKTQPGSLPFDEILYCNIGNPQSLGQQPVTFFREVLALCDHPDLLQREEIKTLFS  barley
::::::: :::::::::::::::::::::: :: :: :::::::::::::::::::::::::::::::::::::::: ::  :: : :::
MAATVAVENLNPKVLKCEYAVRGEIVIHAQHLQQQLQTQPGSLPFDEILYCNIGNPQSLGQQPVTFFREVLALCDHPCLLEKEETKSLFS  P. miliaceum

                                                                                       180
ADSISRAKQILAMIPGRATGAYSHSQGIKGLRDAIASGIASRDGFPANADDIFLTDGASPGVHLMMQLLIRNEKDGILVPIPQYPLYSAS
:: ::::::::  ::::::::::::::::::::::: :::::::::::::::: ::::::::: :::::::::::::: :::::::::::
ADAISRAKQILSTIPGRATGAYSHSQGIKGLRDAIAAGIASRDGFPANADDIFVTDGASPGVHMMMQLLIRNEKDGILCPIPQYPLYSAS

                                                                                       270
IALHGGALVPYYLNESTGWGLETSDVKKQLEDARSRGINVRALVVINPGNPTGQVLAEENQYDIVKFCKNEGLVLLADEVYQENIYVDNK
:::::: :::::: : :::::: :: ::::::::: :: ::::::::::::::::::: :: ::: :::::::::::::::::::::: :
IALHGGTLVPYYLDEKTGWGLEISDLKKQLEDARSKGIDVRALVVINPGNPTGQVLAEDNQCDIVRFCKNEGLVLLADEVYQENIYVDDK
                                                 ●                           ●  ●

                                                                                       360
KFHSFKKIVRSLGYGEEDLPLVSYQSVSKGYYGECGKRGGYFEITGFSAPVREQIYKIASVNLCSNITGQILASLVMNPPKASDESYASY
:: ::::: :: :::: :::::: ::::::::::::::::: :::::::::::::::::::::::::::::::::::::::  ::::: :
KFNSFKKIARSVGYGEDDLPLVSFQSVSKGYYGECGKRGGYMEITGFSAPVREQIYKIASVNLCSNITGQILASLVMNPPKVGDESYAAY
                         ●  ●        ●

                                                                                       450
KAEKDGILASLARRAKALEHAFNKLEGITCNEAEGAMYVFPQICLPQKAIEAAKAANKAPDAFYALRLLESTGIVVVPGSGFGQVPGTWH
:::::::: :::::::::: ::: :::: :: :::::: :::: :: :::::::::::::::::::::::::::::::::::::::::::
KAEKDGILQSLARRAKALEDAFNNLEGISCNKAEGAMYLFPQIHLPKKAIEAAKAANKAPDAFYALRLLESTGIVVVPGSGFGQVPGTWH

                                                                                       482
FRCTILPQEDKIPAVISRFTVFHEAFMSEYRD
 ::::::::::::::: ::  :::::: ::::
IRCTILPQEDKIPAVITRFKAFHEAFMAEYRD
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Figure 3.3. Comparison of the deduced amino acid sequence of barley and *P. miliaceum* (Son and Sugiyama 1992) AlaAT-2. Filled circles indicate conserved residues important for PLP binding.

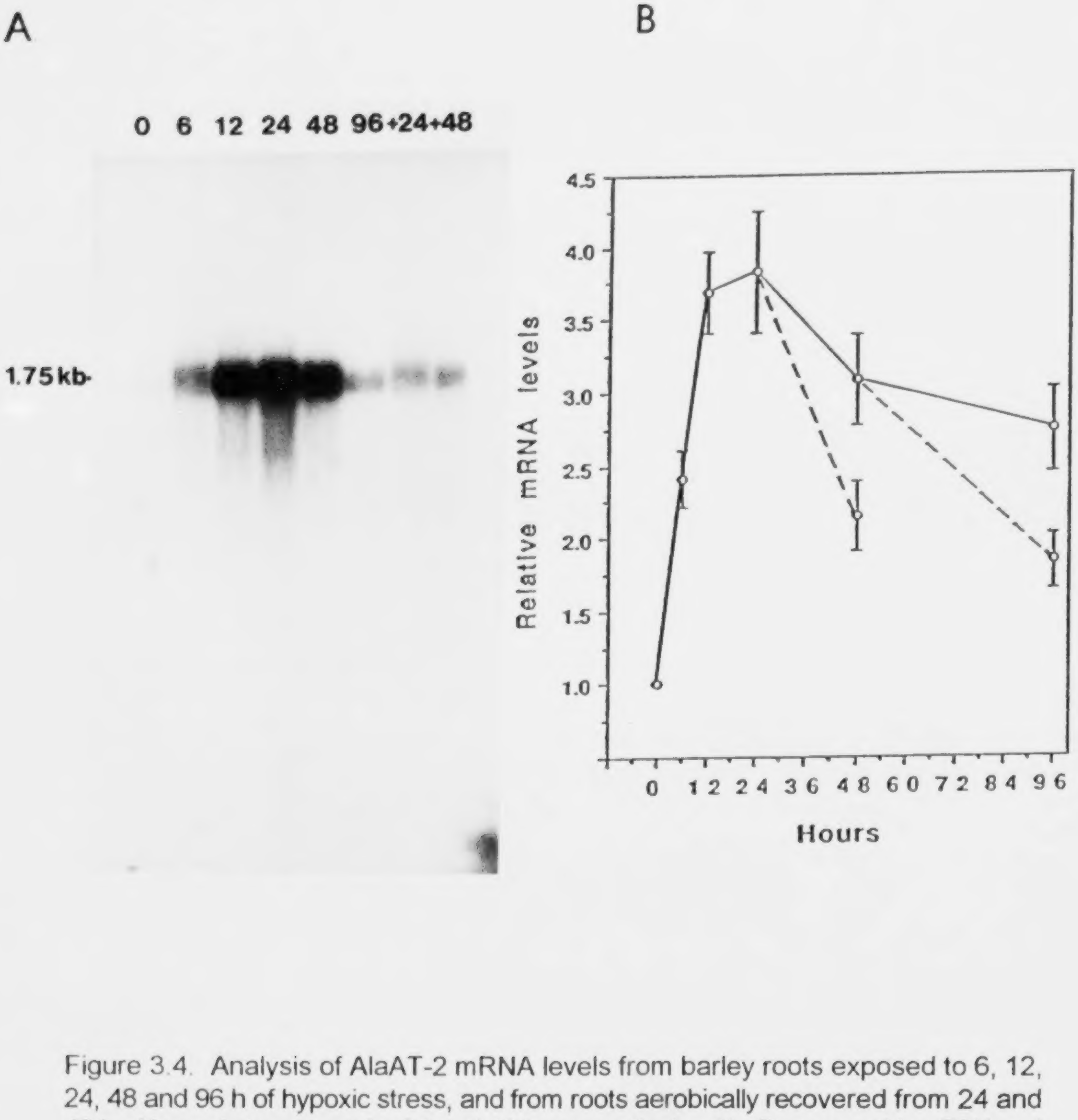

Figure 3.4. Analysis of AlaAT-2 mRNA levels from barley roots exposed to 6, 12, 24, 48 and 96 h of hypoxic stress, and from roots aerobically recovered from 24 and 48 h of hypoxic treatment for 24 and 48 h, respectively. A). Representitive RNA gel blot probed with pCAlaAT3a. B). A curve showing the relative levels of AlaAT mRNA during hypoxic stress. Data are based on slot blot densitometric measurements and were corrected for loading error by normalizing to ribosomal RNA hybridization. Each time point is the mean of data obtained from 2 separate experiments, each replicated twice (±SE). Dotted lines represent data obtained when 24 and 48 h hypoxically induced plants were returned to aerobic conditions for 24 and 48 h, respectively. The mRNA level at t=0 is set at unity.

3a (Fig. 3.4A), indicating that pCAlaAT-3a contained a full length or near full length cDNA clone. The same results were seen when the blot was probed with the 3' untranslated region of pCAlaAT-3a (data not shown). AlaAT mRNA concentrations increased rapidly during the first 6 h of hypoxic stress, and 24 h of hypoxic treatment resulted in an increase in AlaAT message almost 4-fold the concentration found in the control roots (Fig. 3.4B). This is followed by a decrease in message concentration after 48 and 96 h of induction, demonstrating that either transcription rate and/or message stability is reduced upon prolonged exposure to hypoxia. A return to aerobic conditions for 24 or 48 h after hypoxic induction for 24 or 48 h, respectively, shows that there is a gradual return towards aerobic message levels upon oxygenation.

3.3.3 Isolation and partial sequence analysis of a maize AlaAT genomic clone

Approximately 500,000 plaques from a maize genomic library were screened using pCAlaAT-3a as a probe. A genomic clone that strongly hybridized to the AlaAT cDNA probe was isolated, and a restriction map was generated (Fig. 3.5A). The 4.5 kb Hind III/EcoR I fragment (Fig. 3.5A) strongly hybridized to the probe, and was subcloned into pBluescript KS$^+$ (Stratagene) to give the plasmid pGEH4.5. This subclone was then restriction mapped (Fig. 3.5B). The Kpn I/EcoR I 2.1 kb fragment from pGEH4.5 was subcloned into pBluescript KS$^+$ and designated pGKE2.1 (Fig. 3.5C). A partial sequence analysis was performed to determine the degree of sequence similarity between the coding region of this gene and that of the

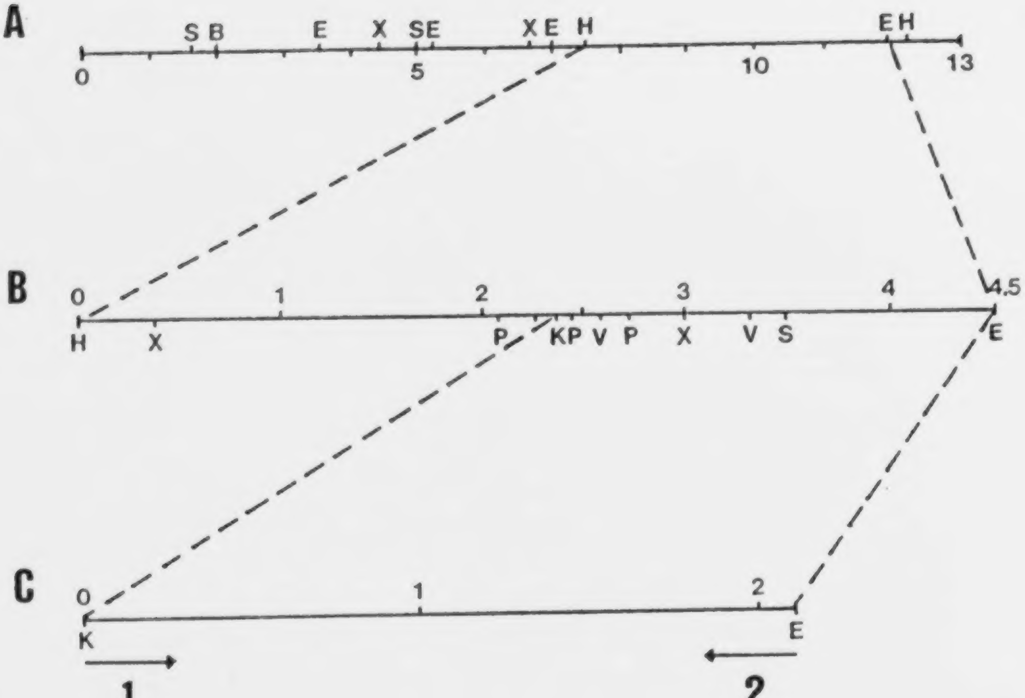

Figure 3.5. Restriction maps of a putative maize AlaAT genomic DNA clone. A) Map of the 13-kb lambda clone. B) Subclone of the 4.5 kb Hind III/EcoR I fragment (pGHE4.5). C) Subclone of the 2.1 kb Kpn I/EcoR I fragment of pGHE4.5 (pGKE2.1). Arrows indicate regions where sequence information was obtained. Numbers indicate distance from the left end of each molecule as shown in kb. Letters are abbreviations: S, Sma I; B, Bam HI; E, EcoR I; X, Xba I; H, Hind III; V, EcoR V; P, Pst I; K, Kpn I.

barley and millet AlaAT cDNAs. The sequence of the outer ends of the subclone was determined for 263 nucleotides from the left end of the subclone (Sequence 1) and 216 nucleotides from the right end (Sequence 2, Fig. 3.5C, 3.6). Both of these sequences appear to cover intron and exon regions of the AlaAT gene when compared to the AlaAT cDNA sequence of barley and millet (Fig. 3.6). The putative intron-exon boundary in sequence 1 and 2 both contain plant consensus intron splice site dinucleotides (Hanley and Schuler 1988) at the intron/exon junction (Fig. 3.6). The putative exon sequence of Sequence 1 and 2 combined is 95.1% (212/223) identical to the corresponding millet cDNA sequence and 87.9% (196/223) identical to the barley cDNA sequence. The 5' sequence data (Sequence 1) is near the 5' end of both the barley and millet cDNAs (300 bp from the 5' end of the cDNA), indicating that pGEH4.5 (Fig. 3.5B) would likely contain the promoter region of this gene.

3.3.4 Gel electrophoresis and immunological detection of the hypoxically inducible AlaAT

Figure 3.7A shows the three AlaAT isoforms found in barley root tissue. AlaAT-2 is the hypoxically inducible isoform of AlaAT in barley root tissue (Chapter 2). However, there is no evidence that the AlaAT-2 activity band intensity increases in hypoxically induced root samples of either barley or maize when run on non-denaturing gels, even though spectrophotometric assays of crude cell extracts indicate that AlaAT activity does increase (Good and Crosby 1989b, Watson et al.

A

```
AGGTTCTTGCCCTTTGTGATCATCCAGACCTGTTGCAAAGAGAGGAAATC  349     barley
:::::::::: ::::::::::::::   ::: ::: ::: :::::::: :
AGGTTCTTGCTCTTTGTGATCATCCGTGCCTCTTGGAAAAAGAGGAAACC   50     maize
::::::::::::::::::::::::: ::: : :::::::::::::::::
AGGTTCTTGCTCTTTGTGATCATCCATGCTTGTTGGAAAAAGAGGAAACT  349     millet


AAAACATTGTTCAG                                      363
::: ::::::::::**
AAATCATTGTTCAGGTGAATATCATCATCATTCAATTTACAGAACTCCTA  100
::::::::::::::
AAATCATTGTTCAG                                      363


AATTCAGAGATCTAGTGTGTGATATCCTGCTATTTTTCCCAACTTCAGTG  150

TCAAAGCAACCTAAAAATTCTAAAAAATACAGAGATAGGGCAATCTATCT  200

TCCTTTAAAATCAAAGCTGaaaaTTTTCTTTGAAATTAGTAAACATTTAT  250

ATAAATAGTAAAA                                       263
```

B

```
TTATCTGCCGTTTGGTCAAGTAACTGGCTTATCCAAAAGATTTTGCTGCT   50


        GATATTATGGTGAGTGTGGTAAAAGAGGTGGTTACTTTGAGA  1034    barley
      **:::: ::::: ::::::::::::::::: :: ::: : ::::
GCAACCAGGATACTATGGAGAGTGTGGTAAAAGAGGAGGGTACATGGAGA   100    maize
        :::: ::::: :::::::::::::::::::: ::::::::::
        GATATTATGGTGAGTGTGGTAAAAGAGGAGGCTACATGGAGA  1034    millet


TTACTGGCTTCAGTGCTCCAGTAAGAGAGCAGATCTACAAAATAGCATCA  1084
:::::::::::::::::::::::::::::::::::::::: ::::: :::
TTACTGGCTTCAGTGCTCCAGTAAGAGAGCAGATCTACAAGATAGCTTCA   150
::::::::::::::::::: ::::::::::::::::::::::::::::::
TTACTGGCTTCAGTGCTCCCGTAAGAGAGCAGATCTACAAGATAGCTTCA  1084


GTGAACCTATGCTCCAATATCACTGGCCAGATCCTTGCTAGTCTTGTCAT  1134
:::::: : ::::::::::::::::: :: :::::::: :: :: :::::
GTGAACTTGTGCTCCAATATCACTGGTCAAATCCTTGCGAGCCTCGTCAT   200
:::::::: ::::::::::::::::::::::::::::: ::::: :::::
GTGAACTTATGCTCCAATATCACTGGTCAAATCCTTGCCAGCCTTGTCAT  1134


GAACCCACCAAAGGCT                                    1150
::: ::::::::::
GAATCCACCAAAGGTC                                     216
::::::::::::::::
GAATCCACCAAAGGTC                                    1150
```

Figure 3.6. Sequence data from the A) 5' end (Sequence 1) and B) 3' end (Sequence 2) of pGKE2.1 (Fig. 3.4C). Regions of this sequence that align with sequence of the barley (Fig. 3.2) and millet (Son and Sugiyama 1992) AlaAT cDNAs are shown. *, consensus splice site dinucleotides. Lower case letters indicate residues of low confidence identity.

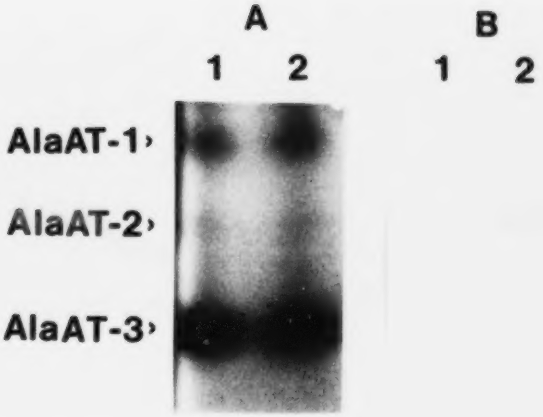

Figure 3.7. A) Nondenaturing gel of crude extracts from 1) aerobic and 2) 96-h hypoxic barley roots stained for AlaAT activity. B) Immunoblot of the nondenaturing gel probed with barley anti-AlaAT-2 antiserum. Each lane was loaded with 12 µg protein.

1992). This isoform stains very poorly when in a homogeneously pure form, indicating that much of its activity is lost on nondenaturing gels (Chapter 2). Figure 3.7A again shows that there is no increase in AlaAT-2 activity on non-denaturing gels, but there is a definite increase in AlaAT-2 protein concentration when the gel is blotted to nitrocellulose and probed with the polyclonal AlaAT-2 antiserum (Fig. 3.7B). Figure 3.7B also demonstrates that the AlaAT antiserum is specific to AlaAT-2 only, as neither AlaAT-1 nor AlaAT-3 were stained in these immunoblots. When hypoxic barley root extract was immunotitrated with AlaAT-2 antiserum, 15, 50 and 97% of the spectrophotometrically measured AlaAT activity was removed when 0.5, 5 and 50 μL of serum, respectively, was incubated with 200 μL of extract. This data indicates that AlaAT-1 and AlaAT-3 possess only minor AlaAT activity relative to AlaAT-2. When the supernatant of immunotitrations was run on nondenaturing gels, the AlaAT-2 activity band disappears when as little as 0.5 μL of serum was added to 200 μL of crude root extract (Fig. 3.8A). When these gels are blotted to nitrocellulose, immunodetection shows that AlaAT-2 protein decreases linearly when 0.5, 1, 2.5 and 5 μL of serum was added (Fig. 3.8B). Although these bands are faint in the photograph, the decrease in band intensity is much clearer in the original blot. When 50 μL of serum is added to 200 μL of root extract, AlaAT-2 is not immunologically detectable at all (data not shown). The AlaAT-1 and AlaAT-3 activity bands are not affected by immunotitration.

Immunoblots of SDS-PAGE gels show that AlaAT-2 protein concentrations increase approximately 6-fold in barley root tissue after 96 h of hypoxia, when

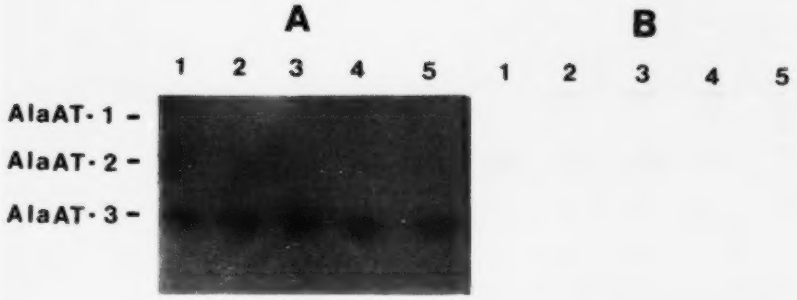

Figure 3.8. A) Non-denaturing gel of the supernatant of crude extracts following immunotitration. Extracts loaded into the lanes were first immunotitrated overnight with 0 μL (lane 1), 0.5 μL (lane 2), 1.0 μL (lane 3), 2.5 μL (lane 4) and 5.0 μL (lane 5) of anti-AlaAT antiserum in 200 μL of crude extract. The band intensities of the AlaAT isoforms are low, likely due to activity loss in the overnight incubation of these preparations. B) Immunoblot of the non-denaturing gel probed with barley anti-AlaAT antiserum. The partial band located at the top of the blot was likely due to cross reaction of the antiserum with the antibody which was added to the sample. This band was not visible in the control lane (lane 1).

compared to aerobic concentrations. This is similar to the increase in total AlaAT activity in hypoxic root tissue (Fig. 3.9). When plants exposed to hypoxia for 24 or 48 h are returned to aerobic conditions for 24 or 48 h, respectively, the amount of AlaAT-2 protein remained high, again consistent with the level of total AlaAT activity (Fig. 3.9). These data again indicate that the majority of root AlaAT activity is AlaAT-2. Fig. 3.4B and Fig. 3.9 show that there is a similar induction pattern of AlaAT-2 mRNA and protein up to 24 h of induction, however after 24 h of induction, protein levels continue to increase linearly , whereas mRNA levels decrease.

3.3.5. Anaerobic induction of P. miliaceum AlaAT

When *P. miliaceum* roots are exposed to hypoxic conditions for 4 d, there is an increase in AlaAT specific activity from 2.95 ± 0.23 IU/mg protein to 7.98 ± 0.11 IU/mg protein when measured spectrophotometrically. This increase (>2 fold) is less than that found in barley (4 to 6-fold, Good and Crosby 1989b), but is similar to that of maize and wheat (>2 fold increase over 5 d of induction, [Good and Crosby 1989b]), and is seen as an increase in AlaAT-2 band intensity on nondenaturing gels (Fig. 3.10A). Native gel immunoblots probed with *P. miliaceum* AlaAT-2 antiserum also show a similar increase in the intensity of the AlaAT-2 protein band in hypoxic samples compared to aerobic controls (Fig. 3.10B). *P. miliaceum* AlaAT-2 maintains its activity on nondenaturing gels much better than barley AlaAT-2. This is seen when root samples containing equal amounts of AlaAT activity from these two species, are run next to each other on AlaAT-stained

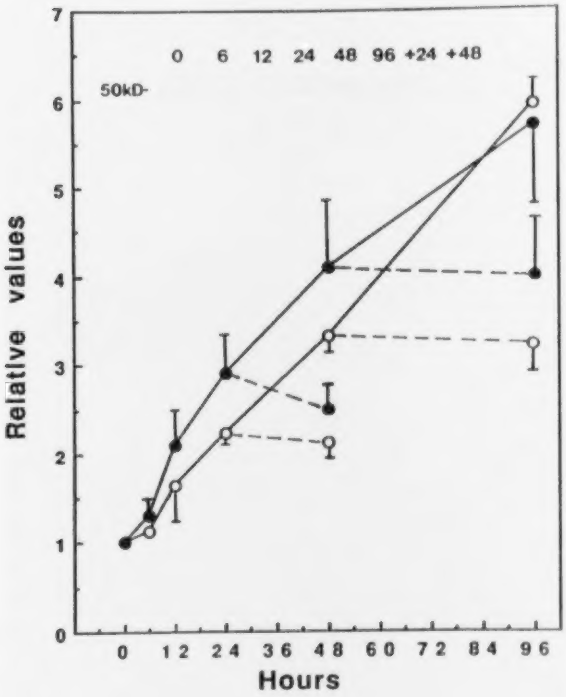

Fig. 3.9. Comparison of AlaAT activity (o) and AlaAT-2 protein (•) concentration in barley roots exposed to hypoxic conditions. Activity was determined spectrophotometrically and protein concentrations were determined by densitometry of SDS immunoblots. Time points are as in Fig. 3. Each time point is the mean of data obtained from 2 separate experiments, each replicated twice (± SE). Inset shows an SDS-PAGE immunoblot probed with barley anti-AlaAT-2 antiserum. Dotted lines represent data obtained when 24 and 48 h hypoxically induced plants were returned to aerobic conditions for 24 and 48 h, respectively. Each lane was loaded with 5 ug protein.

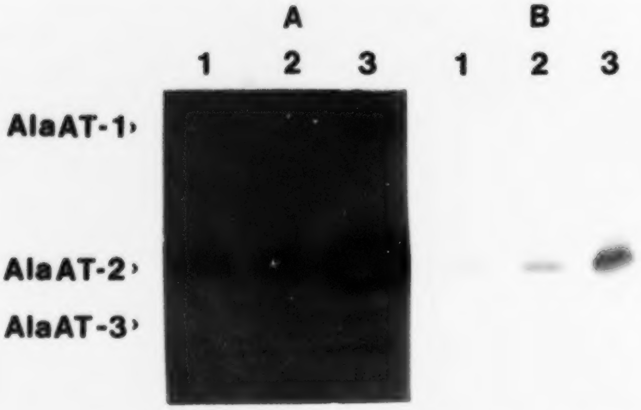

Fig. 3.10. A) Nondenaturing gel of crude extracts from 1) aerobic roots 2) 96-h hypoxic roots and 3) green leaves of *P. miliaceum* stained for AlaAT activity. B) Immunoblot of the nondenaturing gel probed with *P. miliaceum* anti-AlaAT-2 antiserum. Protein loadings were 3.5 μg for root samples and 7.0 μg for leaf.

nondenaturing gels (data not shown). We have attempted several modifications of the gel running and staining procedures, including the addition of PLP to both the barley root extracts and the gel, and have been unable to better retain the activity of barley AlaAT in any of these experiments.

3.3.6. Leaf AlaAT expression

AlaAT-2 was previously shown to be the light inducible AlaAT in *P. miliaceum* leaves (Son et al. 1991), and this leaf isoform has the same electrophoretic ability as *P. miliaceum* root AlaAT-2 on non-denaturing gels (Fig. 3.10A, B). SDS-PAGE immunoblots show that the hypoxically induced root AlaAT-2 and the light induced leaf AlaAT-2 both migrate as the same 50 kD band (data not shown). These results suggest that root and leaf AlaAT-2 are enzymes encoded by the same gene.

When etiolated barley seedlings were exposed to light, there was a slight increase in AlaAT specific activity in leaf tissue, from 0.70 ± 0.03 IU/mg protein in etiolated seedlings to 1.08 ± 0.13 IU/mg protein in 4-day light induced seedlings.

3.4 Discussion

We have isolated a barley root cDNA which contains the entire coding region of AlaAT, and has a 90% deduced amino acid identity to that of a light inducible AlaAT-2 cDNA from *P. miliaceum*. That this barley root cDNA likely codes for the hypoxically induced AlaAT-2 is based on the following results. First, the amino acid sequence of the amino termini of 2 peptides of the hypoxically induced AlaAT-2 of barley roots (52 residues in total) matches exactly the amino acid sequence deduced from corresponding regions of pCAlaAT-3a (Fig. 3.2). Second, the molecular mass of the protein sequence (52,885 Da) predicted by the large open reading frame of pCAlaAT-3a, is similar to that of the purified hypoxically induced barley AlaAT (50 kDa, Chapter 2). Third, RNA gel blots probed with a portion of the 3' untranslated region of pCAlaAT-3a demonstrate an increase in AlaAT mRNA levels in hypoxically induced barley root tissue. Finally, since pCAlaAT was isolated from an hypoxically induced barley root cDNA library, and all the positive clones are apparently truncated versions of the same cDNA, then pCAlaAT is likely to contain the cDNA coding for the hypoxically induced AlaAT-2.

RNA and protein blots indicate that the increase in barley root AlaAT activity is, at least partially, due to an increase in AlaAT-2 mRNA concentration and that the activity increase is directly correlated to an increase in the amount of AlaAT-2 protein present. The rapid increase in AlaAT mRNA concentration within 12 h of hypoxia is similar to that of other ANP mRNAs (Gerlach et al. 1982, Hondred and Hanson 1990, Russell and Sachs 1989), however, the 4-fold increase of AlaAT-2 mRNA upon exposure to anoxic conditions is not as high as *Adh-1* mRNA accumulation in maize (~50-fold, [Gerlach et al. 1982]) or barley LDH mRNA (10-fold, [Hondred and Hanson 1990]). This is likely due to the high constitutive level of AlaAT-2 mRNA in barley. In maize roots, ANPs such as aldolase (Dennis et al. 1988) and enolase (Lal et al. 1991) show a similar degree of hypoxic induction as AlaAT of barley roots. These enzymes also have a relatively high basal level of activity. The high constitutive level of AlaAT-2 expression is likely important for the amino acid biosynthetic function of this enzyme under non-stress conditions. AlaAT-2 mRNA concentration begins to drop off after 48 and 96 h of hypoxic stress, whereas AlaAT protein concentration continues to increase during this time period. This indicates that AlaAT-2 protein is probably quite stable under hypoxic conditions. Alternatively, the synthesis of AlaAT-2 protein may proceed at a slower rate during the initial hours of hypoxic stress and then increase in rate after longer periods of hypoxia. When compared to aerobic conditions, ADH and PDC turn over slowly both under hypoxic conditions and subsequent exposure to aerobic conditions (Lazlo and St. Lawrence 1983). AlaAT may show a similar response here.

The anti-AlaAT polyclonal antiserum raised against AlaAT-2 was specific to that isoform (Fig. 3.7B) and immunotitration of crude root extracts eliminates AlaAT-2 only, as seen in SDS-PAGE immunoblots (Fig. 3.8B). Anti-AlaAT-2 antiserum raised against *P. miliaceum* AlaAT-2 was specific only to that particular isoform as well (Fig. 3.10B, Son et al. 1991). This would suggest that AlaAT-2 is encoded by a gene not closely related to the genes coding for AlaAT-1 and 3. The single band seen in RNA gel blots also supports this prediction. The apparent divergence of AlaAT-1 and AlaAT-3 from AlaAT-2 raises the possibility that they are, in fact, aminotransferases of a different type. Aminotransferases in plants are known to exhibit broad substrate specificities (Givan 1980), and thus AlaAT-1 and AlaAT-3 may be aminotransferases possessing only minor AlaAT activities.

AlaAT-2 activity in *P. miliaceum* increases dramatically upon de-etiolation (Son et al. 1991). We have shown that the AlaAT-2 specific activity in roots of *P. miliaceum* also increases more than 2-fold upon exposure to hypoxic stress. This suggests that the same AlaAT gene is induced by hypoxia in roots and light in leaves of *P. miliaceum*. To our knowledge, this would be the only ANP which also significantly increases in activity upon exposure to light. Exposure of etiolated barley leaves to light results in only a small increase in specific activity of AlaAT (~1.5-fold), compared to the significant increase in *P. miliaceum* activity under the same conditions (~25-fold, [Son et al. 1991]). Moreover, green *P. miliaceum* leaves contain ~30-fold more AlaAT activity than green barley leaves (data not shown). This difference in light induction of AlaAT-2 activity is most certainly a result of the type of photosynthesis these two species employ. *P. miliaceum*, a C_4 plant, utilizes the NAD-malic enzyme type where AlaAT has a significant role in shuttling carbon skeletons between the bundle sheath and mesophyll cells, whereas barley utilizes C_3 photosynthesis in which AlaAT is thought to have no function (Hatch 1987).

The putative hypoxically inducible maize AlaAT gene (Fig. 3.5, 3.6) contains exon sequences very similar to those of barley and millet. Millet is more closely related to maize than is barley, and the AlaAT sequence data also follow this pattern (95.1% identity between millet and maize versus 87.9% identity between barley and maize). The similarity of the coding regions among these species indicates that the maize genomic clone isolated here likely encodes the hypoxically inducible AlaAT. Further sequence analysis and expression data must be done in order to confirm this hypothesis. Sequence identity of the Adh-1 and Adh-2 gene coding regions in maize compared to barley are 84.4% and 85.3%, respectively (Mitchell et al. 1989), further supporting this hypothesis.

In summary, the increase in AlaAT-2 activity in barley roots exposed to hypoxic stress is a result of an increase in AlaAT-2 mRNA concentration, and possibly an increase in the translation rate of this mRNA and/or increased stability of the AlaAT-2 protein. AlaAT is novel amongst the ANPs studied to date in its function of amino acid biosynthesis. The role of this enzyme in alanine biosynthesis under hypoxic conditions appears universal since concentrations of alanine have been shown to increase under hypoxia in virtually all plant species studied to date (Effer and Ranson 1967, Hoffman et al. 1986, Smith and ap Rees 1979, Streeter and Thompson 1972). Another unique characteristic of this enzyme is seen in *P. miliaceum* where AlaAT-2 activity is inducible by hypoxia in roots and by light in leaves. In addition, the AlaAT-2 gene of *P. miliaceum* has recently been shown to be induced in leaf tissue upon recovery from nitrogen stress (Son et al. 1992) indicating that the expression of AlaAT-2 in plants may exhibit very complex mechanisms of regulation.

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4 Long-term hypoxic metabolism in barley root tissue: products of pyruvate metabolism

4.1 Introduction

Under hypoxic or anaerobic conditions there are changes in the levels of glycolytic intermediates in roots and an increased glycolytic flux (the Pasteur effect; Davies 1980, Faiz-ur-Rahman et al. 1974). The Pasteur effect is believed to occur so that the cell can meet its requirements for ATP despite the much lower efficiency of ATP production by fermentation. The increase in glycolytic flux during hypoxia is accompanied by the accumulation of a number of glycolytic end-products including ethanol, lactate, alanine and various organic acids and amino acids (Effer and Ranson 1967b, Hoffman et al. 1986, Roberts et al. 1984, Roberts et al. 1992). A number of alternative pathways to lactate and ethanol fermentation have been discovered in animals with high resistance to anoxia (Fields 1983). The advantages of these alternative strategies for anaerobic fermentation are: increased ATP production per mole of substrate fermented, production of NAD^+ to provide a net sink for reductant, and maintenance of cellular pH.

A version of this chapter has been published. Good AG and Muench DG (1993) Plant Physiol 101: 1163-1168.

Vanlerberghe et al. (1990) showed that the green alga *Selenastrum minutum* accumulated succinate under anaerobic conditions (discussed in Chapter 1). They demonstrated that the pathway to oxaloacetate that is reduced to succinate is initiated by the transfer of the amino group of aspartate to pyruvate to produce alanine. The advantage of this pathway is that it provides a two-fold increase in ATP yield per hexose as compared to fermentation to ethanol or lactate, while at the same time producing 2 NAD^+. Once the aspartate has been depleted, the OAA is synthesized via carboxylation of phosphoenolpyruvate, to produce 2 ATP and 2 NAD^+ per hexose. Succinate has been shown to accumulate in a number of higher plants under oxygen deficiency, however, in the case of rice this accumulation occurred in the shoots but not the roots (Menegus et al 1988, Menegus et al. 1989).

One of the deficiencies of most previous studies on hypoxic metabolism in plants is that they are restricted to short time intervals, usually a maximum of 12 to 24 h (Effer and Ranson 1967a, 1967b, Hoffman et al. 1986, Roberts et al. 1992, Vanlerberghe et al. 1990). However, since barley and maize can survive 3 to 5 d of hypoxic stress (Harberd and Edwards 1982, Lemke-Keyes and Sachs 1989), it may be that the long-term adaptations to hypoxic stress are most important in determining a plant's sensitivity to flooding.

In this study we look at the long-term effects of hypoxia on glycolytic metabolism in order to determine how they differ from short-term effects. We show that, although a number of metabolic products such as lactate, alanine, malate and succinate accumulate during short-term hypoxia, they do not continue to increase during long-term hypoxia. In addition we studied the effect of reducing the level of available nitrogen on the accumulation of metabolites, the production of metabolites in an *Adh1*$^-$ mutant, and the expression of enzymes involved in oxaloacetate and succinate production.

4.2 Materials and methods

4.2.1 Plant materials and growth conditions

Barley seeds (*Hordeum vulgare* L. cv Himalaya and an *Adh1*$^-$ mutant isolated by Harberd and Edwards [1982]) were surface sterilized in 1% NaOCl, rinsed with water and planted in vermiculite. Plants were grown and hypoxically treated as described in Chapter 2.

4.2.2 Nutrient solutions and media experiments

To determine the amounts of metabolites released by the roots, plants were grown with their roots submerged in 30 mL test tubes plugged with cotton and containing half-strength Hoagland solution plus 70 μg/mL chloramphenicol to limit bacterial growth. Plants were transferred to test tubes containing fresh media (aerobic or hypoxic) every 24 h. Therefore, the measure of metabolite concentrations in the medium reflects the cumulative measure over a series of 24 h periods. The solutions were maintained aerobic or hypoxic by slowly bubbling air or N_2 through a fine-bore pipette into the solution. By adding known amounts of ethanol to the test tubes, it was determined that 70 to 85% of the ethanol remained in solution over a 24 h period. The media was assayed directly for the various metabolite levels. Plants used in the nitrate-free experiments were transferred from the half-strength Hoaglands solution into the nitrate-free media (See Appendix 1) upon the start of hypoxic induction.

4.2.3 Enzyme assays

Enzyme extractions were carried out as described in Chapter 2. ADH assays were performed spectrophotometrically in the ethanol → acetaldehyde direction in a 1 mL reaction mix containing 0.1 M Tris-HCl (pH 9.0), 1 mM NAD, 100 μM ethanol and 50 μl enzyme extract. AspAT and MDH activity were assayed by monitoring the oxidation of NADH at 340 nm as described by Griffith and Vance (1989) and Savithiry et al. (1992) respectively. The AspAT assay buffer contained 0.1 M Tris-HCl (pH 8.0), 0.1 mM NADH, 10 mM 2-oxoglutarate, 10 U MDH, 20 mM aspartate and 50 μL enzyme extract to a final volume of 1 mL. The MDH assay buffer contained 0.1 M Tris-HCl, 0.1 mM NADH, 6 mM oxaloacetate and 20 μL enzyme extract to a final volume of 1 mL. Alanine dehydrogenase (AlaDH) activity was assayed at 23°C monitoring the change in OD at 340 nm. AlaDH assays were run in both the forward and reverse directions at different pH's (pH 7,8,9,10) using oxidized and reduced forms of NAD^+ and $NADP^+$. Assay mixtures for the forward (aminating) reaction consisted of 0.1 M Tris-HCl, 0.03 µmol NADH or NADPH, 3 mM

Na-pyruvate and 200 μL of enzyme extract. To initiate the reaction, 0.1 mM NH_4Cl was added to the reaction. The reverse (deaminating) reaction contained 0.1 M Tris-HCl, 1 mM NAD^+ or $NADP^+$ and 200 μL of enzyme extract. The reaction was started by adding 25 mM alanine. In all cases the assay buffers were tested using bacterial AlaDH (Sigma, A1638).

4.2.4 Extraction and assay of metabolites

Root tissue (1 gm) was weighed and then immediately ground in an ice cold mortar and pestle with 5 mL of 0.6 M $HClO_4$ and a small amount of sand. The extract was then microcentrifuged, the supernatant transferred to a new tube, and the pH of the supernatant adjusted to 4.5 with 3 M KOH. The extract was then recentrifuged and the supernatant collected, sealed in microcentrifuge tubes and stored at -70°C until it was assayed. Alanine, aspartate, ethanol, lactate, malate, pyruvate and succinate concentrations were assayed enzymatically according to Bergmeyer (1974; see Appendix 2).

4.3 Results

4.3.1 Enzyme induction

The effect of hypoxia on ADH, AspAT and MDH activity in barley roots of the wild-type Himalaya cultivar is shown in Figure 4.1. ADH activity increased several-fold over the 4 d of hypoxic induction as has been shown previously (Good and

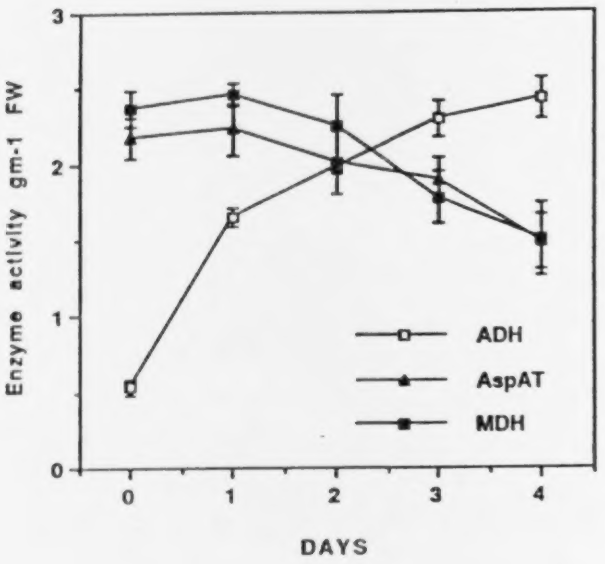

Figure 4.1. Induction of ADH, AspAT and MDH activity in hypoxic roots. Plants were sparged with air until 2 weeks old; N_2 then replaced air for the N_2 and NO_3^- plants. Samples were removed for analysis at daily intervals. Enzyme activities are expressed as IU/gm fresh wieght with the exception of MDH which is IU x 10^{-1}/gm FW. Activities are the mean of three plants, bars represent the standard error (SE).

Crosby 1989a). In contrast, AspAT and MDH activity were not hypoxically induced in roots. Both enzymes remained constant in activity during the first 24 h of induction then decreased for the next 3 d. Removing NO_3^- from the nutrient solution had no effect on the induction pattern of either AspAT or MDH activities (data not shown). In bacteria and algae, alanine can be formed from pyruvate either by AlaAT or alanine dehydrogenase (AlaDH, Goodwin and Mercer 1983, Rowell and Stewart 1976). We tested the possibility that AlaDH was converting pyruvate to alanine by assaying for AlaDH activity in aerobic and hypoxic root tissue extracts. Using different pH's (pH 7, 8, 9, 10) and different cofactors (NAD^+, $NADP^+$) in forward and reverse reactions, we were unable to detect AlaDH activity in any of the tissue extracts including leaf extracts.

4.3.2 Fermentation products

4.3.2.1 Pyruvate, lactate, ethanol

With the onset of hypoxia there is a rapid, though slight, increase in pyruvate concentration in root tissue. In the presence or absence of nitrate, the level of pyruvate in the hypoxic wild type plants rose to twice that of the aerobic control within 24 h and remained unchanged for the remaining 72 h (Fig 4.2). The level of pyruvate in the *Adh*⁻ plants increased more rapidly than in wild-type suggesting that, in the absence of a functional *Adh1* gene (Harberd and Edwards 1982), the decreased production of ethanol results in an accumulation of pyruvate (Fig 4.2).

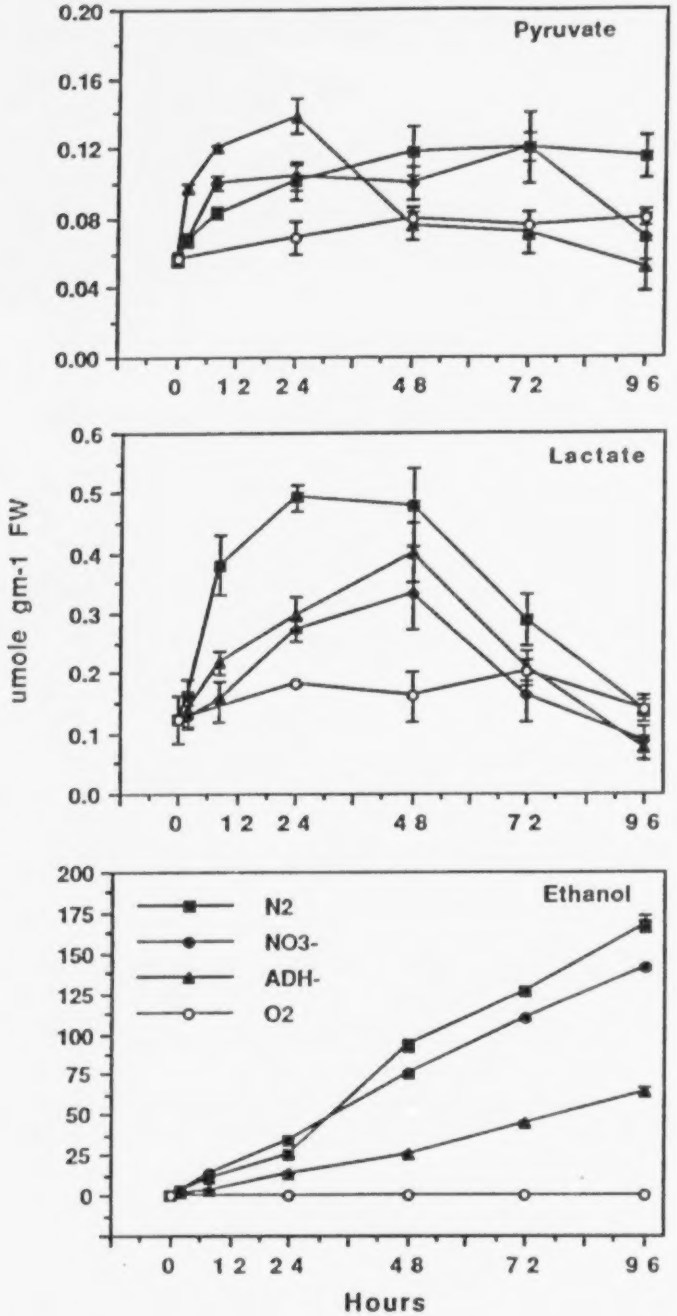

Figure 4.2. Pyruvate, lactate and ethanol concentrations in hypoxically induced barley root tissue. Metabolite concentrations were measured in root tissue or media ("Materials and Methods") from plants sparged with air (o) or N_2 (■) or grown in a nitrate-free nutrient solution sparged with N_2 (●). ▲, Adh^- plants sparged with nitrogen. Values are the mean of three plants, bars represent the SE.

However after 24 hrs the level of pyruvate began to decrease until it was at or below its concentration in the control plants (Fig 4.2).

Lactate concentration increased 4-fold within the first 24 h of hypoxia (Fig. 4.2). Subsequent to that, the concentration decreased until it had returned to that of the control plants at 96 h. Lactate accumulated more slowly in plants grown in the nitrate-free media and in *Adh*$^{-}$ plants. However, the pattern of rapid increase followed by a gradual decline in lactate levels remained the same.

The total ethanol accumulation (tissue and media) under hypoxic conditions is higher than any other metabolite studied (Fig 4.2). Ethanol accumulated at an average rate of 42 µmol gm/FW/day. In the absence of a nitrogen source, the rate of ethanol accumulation was slightly lower than for plants grown in Hoagland's solution. The concentration of ethanol production by the *Adh*$^{-}$ plants was 16 µmol/gm FW/day, less than 40% of that found in the wild type plants. While none of the other metabolites were detected in the culture medium, almost all of the ethanol that accumulated during hypoxia was present in the media after diffusing out of the root tissue. The concentration of ethanol in root tissue was at most 2% of that found in the media. Ethanol was the predominant product of long-term hypoxic glycolysis, occurring at levels up to 50 times higher than that of any of the other metabolites (Fig. 4.2).

4.3.2.2 Alanine and Aspartate

There is a rapid increase in alanine concentration during the first 24 h of hypoxia, followed by a levelling off through to 96 h (Fig 4.3). Aspartate concentrations decreased rapidly almost immediately after exposure to hypoxic conditions (Fig 4.3). Vanlerberghe et al. (1990) have argued that during the early stages of hypoxia the amino group of aspartate is conserved by transaminating pyruvate to alanine. Similarly we found that the decrease in aspartate over the first 8 h can account for the increase in alanine, since during this time a stoichiometry of approximately 1.08 mol of aspartate depleted per mole of alanine accumulated was observed. After 8 h of hypoxia, the level of alanine continued to increase another 2-fold. The level of aspartate, however, did not decrease further after the first 8 h, instead it levelled off and then rose slowly (Fig 4.3). This pattern of aspartate depletion was similar for the *Adh*$^-$ plants and plants in the nitrate-free medium.

Although the levels of alanine in the *Adh*$^-$ plants rose more slowly than in the wild-type plants, they eventually reached similar concentrations near the end of the induction period (Fig 4.3). The plants grown in a nitrate-free medium had levels of alanine that continued to increase for 48 h but then proceeded to decrease to control levels. This clearly indicates that after 8 h, the amino group of alanine is no longer provided by aspartate. In order to maintain alanine levels, a medium that contains a supply of nitrogen is required.

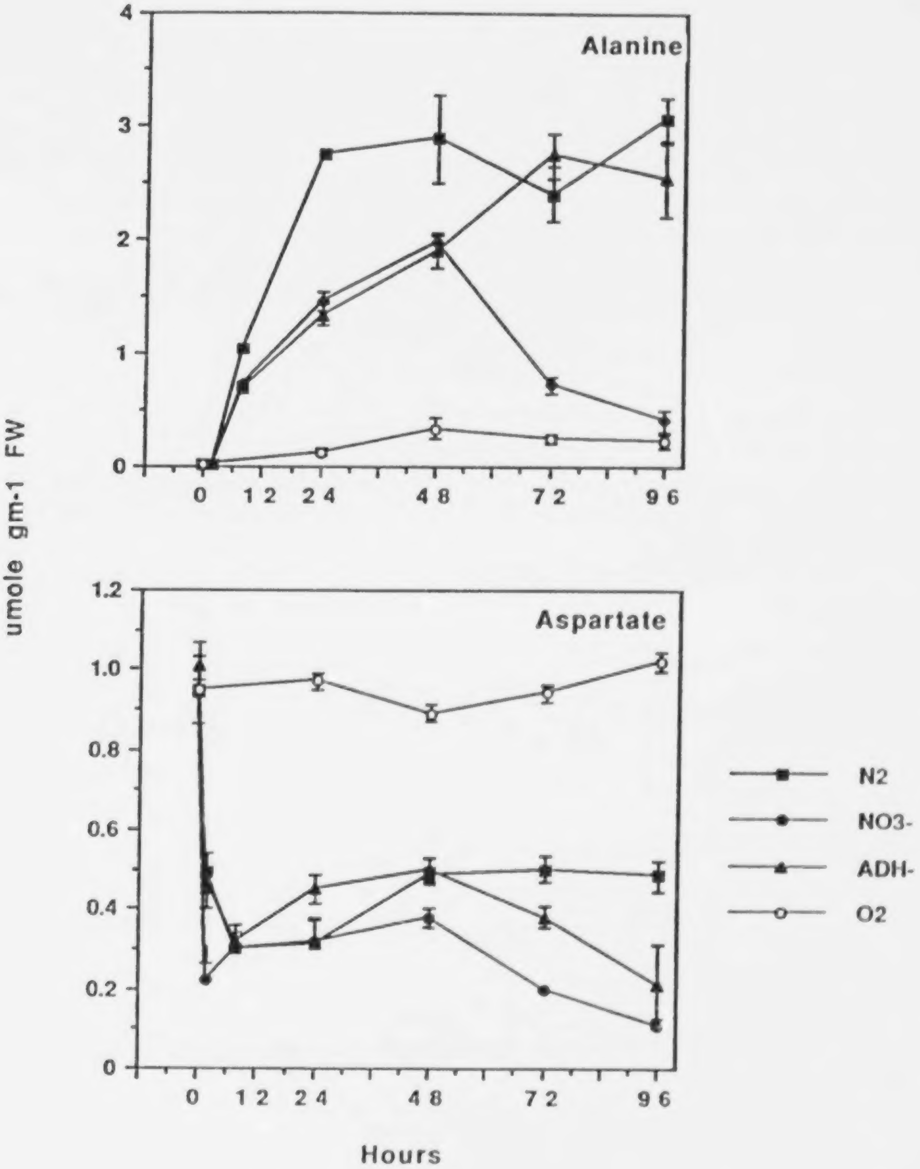

Figure 4.3. Alanine and aspartate concentrations in hypoxically induced barley root tissue. Ala and Asp concentrations were measured in root tissue from plants sparged with air (o) or N_2 (■) or grown in a nitrate-free nutrient solution sparged with N_2 (●). ▲, Adh⁻ plants sparged with nitrogen. Values are the mean of three plants, bars represent the SE.

4.3.2.3 Malate and Succinate

Malate showed an initial increase during the first 8 h of hypoxia, followed by a gradual long-term decline (Fig 4.4). This occurred for both genotypes, and in the presence or absence of NO_3^-. Succinate increased approximately 1.5-fold during the first 8 h, followed by a gradual decrease back to control levels after 24 h. Thereafter, the concentration of succinate remained stable (Fig 4.4). The *Adh*$^-$ plants initially had a higher level of succinate, which increased during the first 8 h of hypoxia and then gradually declined to a level similar to the wild-type plants.

4.4 Discussion

4.4.1 Enzyme induction

The onset of hypoxia in root tissue has been shown to result in increases of a number of glycolytic enzymes including ADH, LDH and AlaAT (Good and Crosby 1989a, 1989b, Kelley and Freeling 1984a, 1984b). One of the interesting observations from this work is that while both LDH and AlaAT activity levels continue to accumulate for up to 96 h (Good and Crosby 1989a, 1989b), the end-products (lactate and alanine) reach their maximum levels after the first 24 h (Fig. 4.2, 4.3). Both AspAT and MDH remained constant in activity during the first 24 h, followed by a long-term decline in activity levels. The end-products aspartate and malate, however, are not maintained at a steady state. Under hypoxic conditions, aspartate concentration drops rapidly, whereas the concentration of malate showed

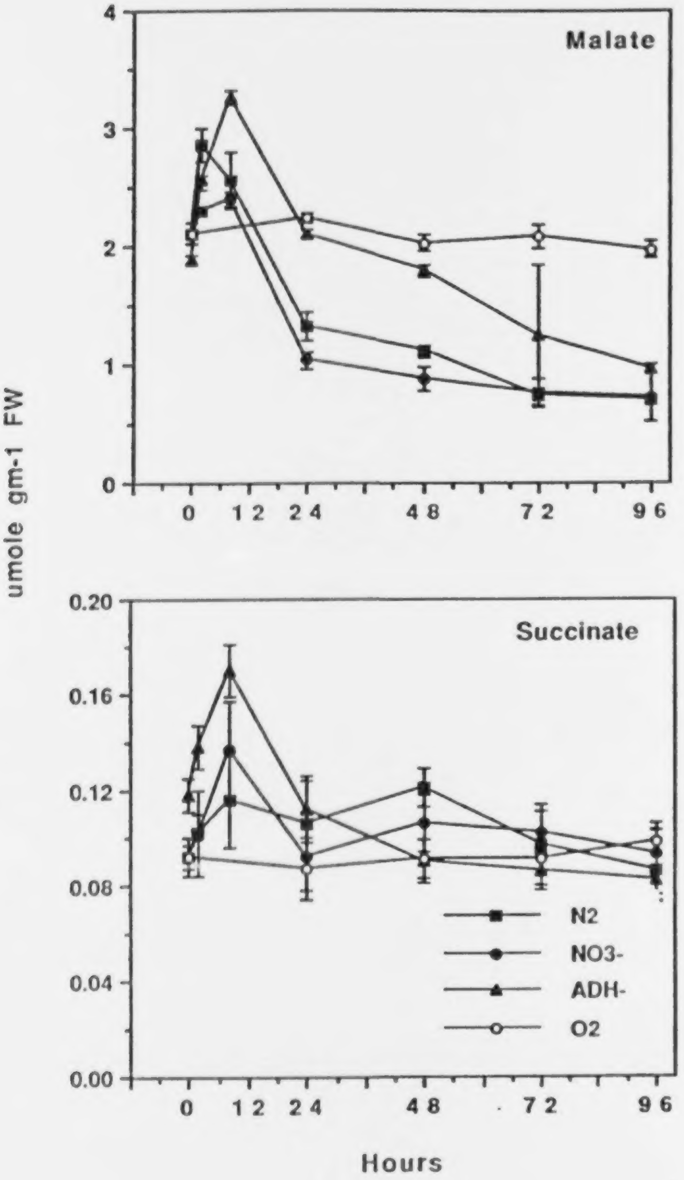

Figure 4.4. Malate and succinate concentrations in hypoxically induced barley root tissue. Malate and succinate concentrations were measured in root tissue from plants sparged with air (○) or N_2 (■) or grown in a nitrate-free nutrient solution sparged with N_2 (●). ▲, Adh⁻ plants sparged with nitrogen. Values are the mean of three plants, bars represent the SE.

a transient increase followed by a steady decrease. The enzyme involved in the synthesis of succinate, succinate dehydrogenase, is not induced under hypoxic conditions (Kennedy et al. 1987). ADH activity and ethanol synthesis increase throughout the induction period. These data suggest a correlation between the hypoxic induction of an enzyme and an increase in the level of product for at least the first 24 h of hypoxia. However, AlaAT and LDH do not appear to need further induction under long-term hypoxia, as the products of these enzymes no longer increase in concentration. The evolutionary and physiological significance of this response is not known.

The possibility that alanine is formed from pyruvate by alanine dehydrogenase seems unlikely, since AlaDH activity was undetectable in any of the tissues assayed. While AlaDH occurs in bacteria and algae (Goodwin and Mercer 1983, Rowell and Stewart 1976), to the best of our knowledge, the only report of AlaDH activity in higher plants was in mulberry (Talyshinskii 1983).

4.4.2 Fermentation products

The slight accumulation of pyruvate in hypoxic root tissue presumably results from the Pasteur effect which is an increase in glycolytic rate so that the cell can meet its ATP requirements. The Pasteur effect has been shown to occur in both higher plants and in algae (Faiz-ur-Rahman et al. 1974, Peavey et al. 1983, Vanlerberghe et al. 1989). Associated with this increase in pyruvate is a continuous abundant production of ethanol (Fig. 4.2), most of which moves from the root tissue into the medium. The amount of ethanol produced by the *Adh*$^-$ mutant was only 40% of that produced by the *Adh*$^+$ genotype. This corresponds to the reduction in ADH activity in these plants (Good and Crosby 1989a) indicating that the rate of ethanol accumulation may be determined by the level of ADH activity. Lactate and alanine are also important metabolic end-products during the first 24 h of hypoxia (Fig. 4.2), however they do not accumulate during long-term hypoxia.

The reciprocal changes in alanine and aspartate concentrations during the first 8 h of hypoxia supports the hypothesis of Vanlerberghe et al. (1990). They argue that during short-term hypoxia, deamination of aspartate provides the OAA for reduction to succinate (Fig. 1.4). The amino group of aspartate is conserved by transaminating pyruvate to alanine. We have found a 1:1 relationship between the production of alanine and the depletion of aspartate during the first 8 h of hypoxia. After that point, the level of alanine continued to increase for the next 16 h. Therefore, during this stage of hypoxia, the amino group of alanine clearly does not come from aspartate. Because alanine synthesis requires nitrogen, it has been suggested that the nitrogen status of a tissue may be important in determining whether alanine is a significant fermentation endproduct (Good and Crosby 1989b, Hoffman et al. 1986). Vanlerberghe et al. (1991) and Menegus et al. (1993) have recently shown that under hypoxic conditions almost all of the assimilated nitrogen accumulates in alanine (Fig. 1.5.) Similarly, we found that in the absence of a nitrogen source and under hypoxic conditions, the alanine concentration did not reach that found in plants grown in a nitrogen rich media then decreased after 48

h. Presumably the increase in alanine seen between 8 and 48 h is a result of the endogenous nitrogen supply, since our plants were not maintained in a nitrogen limiting environment prior to hypoxic induction.

Aspartate levels have been shown to decrease dramatically during the onset of hypoxia, both in higher plants and algae (Roberts et al. 1992, Vanlerberghe et al. 1990). We found a similar pattern of aspartate depletion; however, in the wild-type plants the level of aspartate increased slightly during the next 72 h. As with alanine, this increase was dependent on an external nitrogen source.

The pattern of malate and succinate accumulation during short-term hypoxia in higher plants and algae has recently been characterized (Roberts et al. 1992, Vanlerberghe et al. 1990). Malate has been shown in some studies to accumulate during anoxia (Davies 1980) whereas in other studies there has been no evidence of malate as a significant fermentative end-product (Roberts et al. 1992, Vanlerberghe et al. 1991). Consistent with what has been found in maize and *S. minutum*, (Roberts et al. 1992, Vanlerberghe et al. 1991) we found that there is a short-term increase in malate, followed by a long-term decline (Fig. 4.4).

Although it has been argued that succinate accumulation plays a role in resistance to hypoxia (Menegus et al. 1988, Menegus at al. 1989). Roberts et al. (1992) found that succinate does not accumulate in maize root tips. We found a similar pattern, with succinate showing a short-term increase in concentration followed by a long-term decline. Presumably the short-term increase in succinate levels results from the deamination of aspartate. However the fate of all the OAA produced by deamination of aspartate during the early stages of hypoxia is not clear, since the ratio of aspartate depletion to succinate accumulation is not 1:1.

One of the mechanisms by which plants might be able to survive long-term hypoxia would be to use alternative fermentative pathways with a higher yield of ATP per mole of substrate fermented. While it has been argued that the formation of succinate might be such an alternative pathway, our data indicate that, while succinate and malate are short-term products of anaerobic fermentation, in the long-term they are not (Fig 4.4). In addition, none of the enzymes involved in aspartate deamination and succinate production show any form of hypoxic induction (Fig 4.1, Kennedy et al. 1987). The predominant long-term hypoxic product in barley roots is clearly ethanol. It has been argued that LDH and ADH activities do not seem to be directly involved in the mechanisms of resistance to anoxia (Roberts at al. 1989; Xia and Saglio 1992). However, the only known mutation that has been shown to affect survival during hypoxic stress is an Adh^- mutant (Harberd and Edwards 1982).

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5 Hypoxic metabolism in wild rice (*Zizania palustris* L.): enzyme induction and metabolite production

5.1 Introduction

Wild rice (*Zizania* spp. L.) is an aquatic, cross-pollinating, North American cereal which differs from domesticated rice (*Oryza sativa* L.) in many morphological, anatomical and ecological features (Campiranon and Koukkari 1977 and references therein). Wild rice survives naturally in shallow, slow-moving water that has sufficient oxygen. Seeds are produced in the fall, shatter, and generally remain submerged until they germinate in the spring. Successful establishment and survival of wild rice in a flooded environment necessitates that the upper soil layer and overlying water have sufficent oxygen, while stem anatomy allows transport of air to the submerged parts of the plant at the aerial leaf stage (Weir and Dale 1960).

In several aquatic plant species, metabolic and anatomical adaptations have allowed for survival in reduced oxygen environments. Wild rice has the ability to germinate in a gaseous oxygen environment as low as 0.3% (Svare 1960). Campiranon and Koukkari (1977) reported that the germination frequency of wild rice seeds was the same under aerobic and anaerobic conditions. Under anaerobic conditions, shoot growth occurred at a substantially reduced rate relative to aerobic

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conditions, and root formation was completely inhibited even 14 days post-germination. Kordan (1972) discovered a similar response in *Oryza*. Campiranon and Koukkari (1977) have also demonstrated that ADH activity in shoots of wild rice is highly inducible under anaerobic conditions, a response that also is seen in *Oryza* (Cobb and Kennedy 1987).

Since wild rice has the ability to germinate and grow under hypoxic conditions, we were interested in determining the metabolic responses which might be important in conferring tolerance to hypoxia in this genus. We have examined the induction and isozyme patterns of several enzymes of wild rice during hypoxia and have measured the production of metabolites which had previously been shown to change in concentration under hypoxic conditions in other species. The possible role of these enzymes and metabolites in conferring tolerance to hypoxic stress in wild rice is discussed.

5.2 Materials and methods

5.2.1 Plant material and growth conditions

Wild rice (*Zizania palustris* L.) seeds were collected from Northern Saskatchewan, Canada, and stored for several months in water at 4°C. Seeds were germinated and grown in 20-L fish tanks filled with water and sparged with air under a 16 h photoperiod at 21°C (250 µmol photons/m/s photosynthetic photon flux density supplied by white fluorescent lamps). After 10 d, 50% of the plants were submersed in N_2-sparged water, and 2 to 4 plants were subsequently removed for sampling from each tank (O_2 and N_2) at each sample time. Plants were held about three cm below the water surface by one layer of cheesecloth covering the top of a rectangular framed support within the tank. A sheet of plastic wrap was placed on the water surface to maintain low oxygen levels, while still allowing light penetration from above. When sampled, all plants possessed 3 to 4 thin, ribbon-like leaves 5 to 10 cm in length, and one primary and several adventitious roots 7 to 10 cm in length. The coleoptile was not sampled in this study.

5.2.2 Enzyme extraction and assay

Root and leaf tissue was blotted, weighed and then ground in a mortar and pestle in extraction buffer (0.1 M Tris-HCl pH 8.5, 10 mM sodium borate, 15% glycerol, 10 mM dithiothreitol and 5 mg/mL bovine serum albumen) and a small amount of sand. The ADH assays were performed in the ethanol → acetaldehyde direction, as described in Chapter 4. The AlaAT assays were performed in the alanine → pyruvate direction as described in Chapter 2. Since these experiments were performed prior to our finding that the AlaAT activity assay and stain inadvertantly assayed for GDH as well, liquid LDH suspended in ammonium sulphate was used in these experiments. The implications of this are discussed in the "Results" section. The LDH assays were performed in the pyruvate → lactate direction according to Hoffman et al. (1986). The assay mix contained (in a final volume of 1 mL) 0.13 M Tris-HCl (pH 8.0), 0.1 mM NADH, 3 mM 4-methyl-pyrazole, 15 mM sodium pyruvate, and 200 μL enzyme extract. All reactions were assayed spectrophotometrically at a temperature of 23°C at 340 nm to measure the increase or decrease in NADH concentration. All activities are given in international units per gram fresh weight (IU= μmol substrate consumed/min).

5.2.3 *Extraction and assay of metabolites*

Lactate, alanine, aspartate, malate and succinate were extracted from leaf and root tissue by grinding the tissue in 0.6 M perchloric acid ($HClO_4$) as described in Chapter 4. The majority of the ethanol produced by the plant (>95%) was shown to diffuse into the growth medium from the tissue, therefore ethanol concentrations were determined by directly assaying samples of the growth medium. Wild rice plants were totally immersed in test tubes and the hypoxic treatment and sampling were performed as in Chapter 4. All metabolites were assayed enzymatically according to Bergmeyer (1974; Appendix 2).

5.2.4 *Nondenaturing polyacrylamide (PAGE) gel electrophoresis*

Root, leaf and seed enzymes were extracted as above and electrophoresed overnight through 1.5 mm nondenaturing gels at 15 mA and 4°C as described in Chapter 2. Gels were stained for AlaAT activity as described in Chapter 2, using LDH suspended in ammonium sulphate. ADH and LDH activities were stained as described by Brown et al. (1978) and Hanson and Jacobson (1984), respectively. The ADH stain consisted of 50 mL 0.1 M Tris-HCl (pH 8.0), 0.1 M ethanol, 30 mg

NAD$^+$, 20 mg nitroblue tetrazolium chloride (NBT) and 5 mg phenazine methosulphate (PMS). The LDH activity stain consisted of 50 mL 0.15 M Tris-HCl (pH 8.0), 1 gm lithium lactate, 100 mg NAD$^+$, 20 mg $MgCl_2$, 10 mg NBT, 2.5 mg PMS and 20 mg 4-methyl pyrazole. The ADH and LDH gels were stained for 15 to 30 min at 37°C.

5.3 Results

5.3.1 Induction of enzyme activity

Activities of ADH, AlaAT and LDH extracted from root and leaf tissue of wild rice were assayed spectrophotometrically over a 4 d induction period following imposition of hypoxic conditions (Fig. 5.1). In root tissue, the activity of all three enzymes increased under hypoxia. ADH and AlaAT activities increased at similar rates, although AlaAT activity was 5 to 6-fold higher than ADH in the control samples. The increase in LDH activity was not as high as those of ADH and AlaAT. The pattern of enzyme induction in wild rice root tissue is similar to that of barley roots (Hoffman et al. 1986, Good and Crosby 1989a, 1989b). In leaf tissue, ADH and AlaAT again showed a similar increase in activity although more slowly than in root tissue. LDH activity was not induced by hypoxia in leaf tissue.

Although it was determined in Chapter 2 that the AlaAT activity assay and stain also detected GDH activity, these wild rice experiments were done prior to our learning of the non-specific nature of these assays. As a result the AlaAT activities

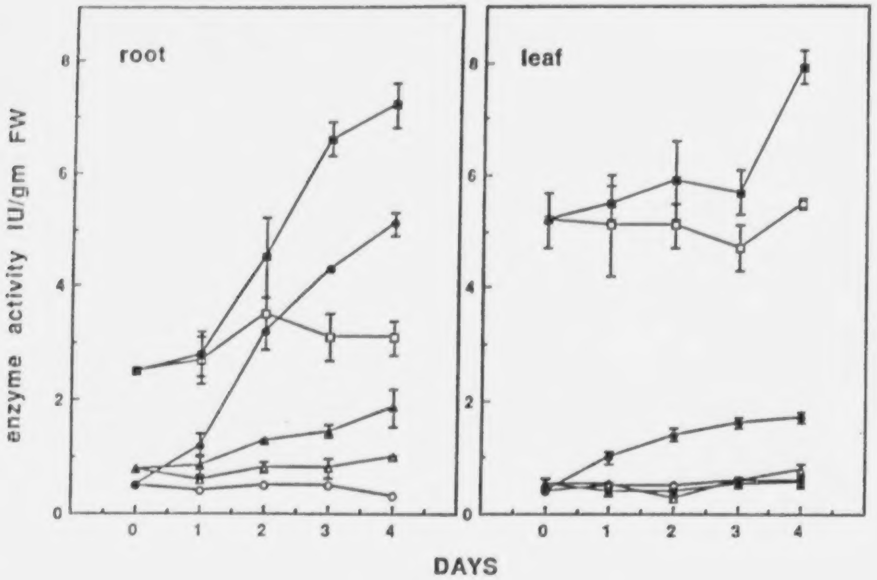

Figure 5.1. The effect of hypoxia on ADH (●), AlaAT (■) and LDH (▲) in root and leaf tissue. Open symbols designate aerobic control plants while closed symbols are hypoxically treated plants. Plants were sparged with air for 10 days; N_2 then replaced air for the hypoxically induced plants. Data points are the means of three plants. Bars represent SE.

presented here also measure GDH activity. Further analysis must be performed to determine if the hypoxic increase in AlaAT activity is due to AlaAT or/and GDH. However, since AlaAT rather than GDH is hypoxically inducible in barley (Chapter 2) it is probable that the activity increase seen in Fig. 5.1 is also AlaAT.

5.3.2 Isozyme profiles of ADH, AlaAT and LDH

The isozyme profiles for ADH, AlaAT and LDH from uninduced and induced root and leaf tissue and from seed are shown in Fig. 5.2. A single isoform of ADH is induced in both root and leaf tissue under hypoxic conditions, whereas three bands of activity are seen in seed (Fig. 5.2). ADH exists as a dimer in plants (Harberd and Edwards 1983). Based on the isozyme pattern, the three bands in the seed sample indicated a random association of the subunits to form either heterodimers or homodimers. These subunits are probably products of two different genes, however it is possible that two different alleles of a single gene are present. Since wild rice is a naturally cross-pollinating species, the frequency of heterozygosity is probably quite high. The number of ADH genes in other plant species varies, as maize has two ADH genes, whereas barley has three and *Arabidopsis* has one (Chang and Meyerowitz 1986, Harberd and Edwards 1983, Freeling 1975). The presence of a single isozyme in root and leaf tissue and the presence of three isozymes in seed is contrary to what has been found in maize and barley. In these species, seed contains only a single isozyme, and root and leaf

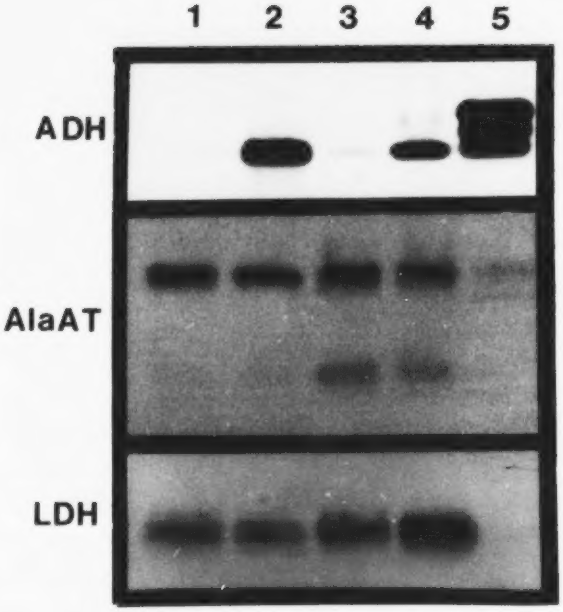

Figure 5.2. Native PAGE of ADH, AlaAT and LDH in different wild rice tissues under aerobic and 4-day hypoxic conditions. Samples loaded were: lane 1, root aerobic; lane 2, root hypoxic; lane 3, leaf aerobic; lane 4, leaf hypoxic; lane 5, seed. The equivalent of tissue loaded per lane was: ADH, 10 mg for root and leaf, 37 mg for seed; AlaAT, 20 mg for root and leaf, 54 mg for seed; LDH, 30 mg for root and leaf, 111 mg for seed.

tissue contain three and six isozymes, respectively (Freeling 1975, Hanson et al. 1984).

Both root and leaf tissue show two bands when stained for AlaAT activity; a more intense, slow migrating band and a less intense, fast migrating band (Fig. 5.2). This isozyme profile shows that, although this enzyme is probably induced under hypoxic conditions in root and leaf tissue when measured spectrophotometrically (Fig. 5.1), a corresponding increase is not seen on native gels. This same effect was observed in both barley and maize (Good and Crosby 1989b, Watson et al. 1992). In Chapter 2 it was determined that there was a loss of AlaAT-2 activity when the enzyme was run on non-denaturing gels. This same effect may explain the lack of increase in any of the AlaAT bands in wild rice as well.

Since the gel stained for AlaAT in Fig. 5.2 would also detect GDH, one or both of the visible bands may be GDH. When this stain was used to stain for AlaAT activity in maize and barley, the most upper (cathodal) band was GDH (Chapter 2, Watson et al. 1992). It is possible that this same band on the AlaAT gel of wild rice (Fig. 5.2) is also GDH, although an AlaAT gel stain lacking ammonium sulfate must be used to confirm this prediction. In seed, AlaAT activity is virtually nonexistent on the native gel as two bands are barely visible. The slow migrating isoform is identical to the root and leaf slow isoforms and may also be GDH, whereas the second band is novel and migrates slightly faster than the slow isoform. Spectrophotometric assays measuring seed AlaAT activity are high (3 IU/gm) suggesting that seed AlaAT activity is reduced on native gels as well. As a result it is probable that root, seed and leaf tissue each express only a single isoform of AlaAT. The seed AlaAT isoform migrates differently than that of leaf and root, and is apparently not the same isoform.

Each LDH banding profile in wild rice root and leaf tissue (Fig. 5.2) shows five bands of activity, two of which stain very intensely. In plants and animals, the LDH holoenzyme is a tetramer composed of the random association of two different gene products (Everse and Kaplan 1973, Hoffman et al. 1986). The LDH zymogram indicates that wild rice either possesses two Ldh genes, or that the plant assayed is heterozygous at a single Ldh locus. Regardless, if both LDH subunits were present equally, a 1:4:6:4:1 LDH isozyme ratio would be predicted (Hoffman et al. 1986). It appears that in wild rice the cathodal LDH isozymes stain more intensely, indicating that the cathodal LDH subunit is expressed at a higher level than the anodal subunit. Figure 5.2 shows that there is no activity in seed on a nondenaturing gel although a very small amount of activity is detected when assayed spectrophotometrically (average of 0.06 ± 0.02 IU/gm).

5.3.3 Metabolite concentrations in seedlings subjected to anaerobic conditions

5.3.3.1. Ethanol and lactate.

Of the metabolites studied, ethanol showed the greatest accumulation under hypoxia. For whole plants, ethanol concentration increased linearly during the first 3 d of hypoxia and then began to plateau during the last day (Fig. 5.3). Lactate

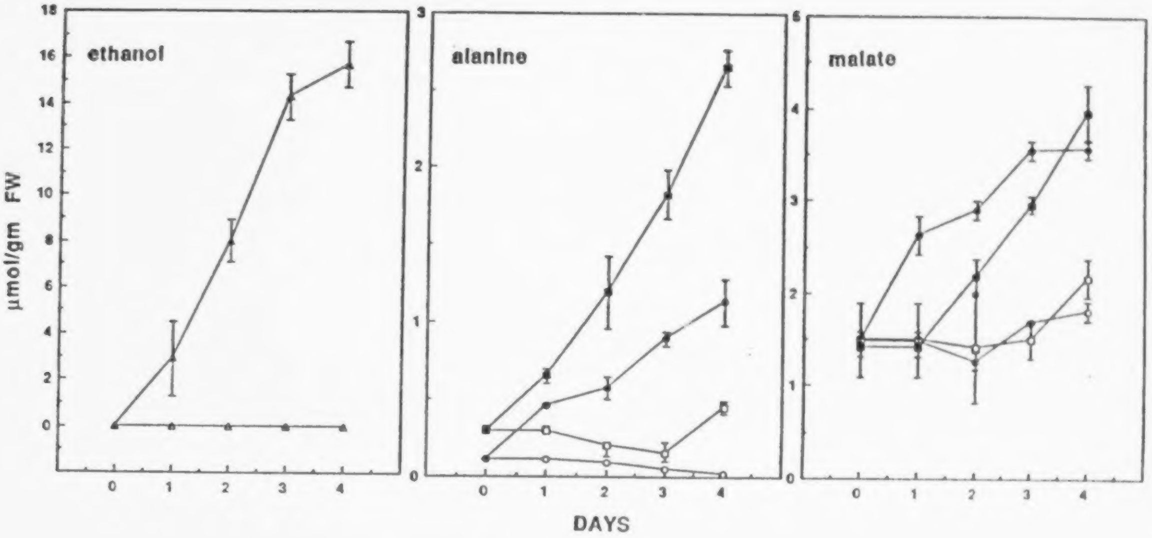

Figure 5.3. Changes in ethanol, alanine and malate concentration in wild rice during hypoxia. Symbols indicate measurements from, (▲) whole plants; (■) leaf; (●) root. Open and closed symbols refer to aerobic and hypoxic treatments, respectively. The data points are the means of three plants. Bars indicate SE.

concentrations were low (0.5-1.0 µmol/gm) and fluctuated within this range in both root and leaf tissue during the induction period (data not shown).

5.3.3.2. Alanine and aspartate.

Alanine accumulated gradually over a 4 d of hypoxia in both root and leaf tissue although leaf tissue produced more than twice as much alanine as root tissue (Fig. 5.3). The concentration of aspartate in both root and leaf tissue remained constant throughout the induction period with an average of 0.1 ± 0.01 µmol/gm (data not shown).

5.3.3.3. Malate and succinate.

Malate concentration increased in a fashion similar to that of alanine, although malate levels were slightly higher (Fig. 5.3). The concentration of succinate in wild rice leaf and root tissue remained low and constant throughout the hypoxic period (average of 0.34 ± 0.02 µmol/gm; data not shown).

5.4 Discussion

The hypoxic induction of ADH activity and the synthesis of ethanol in root and shoot tissues of wild rice implies that ethanolic fermentation play an important role in hypoxic respiration in wild rice. Since ethanol production during hypoxia is high in virtually all plant species, it is not surprising that it is the major product of hypoxic metabolism in wild rice. Studying *Oryza* and *Echinochloa*, Cobb and Kennedy (1987) showed that flood-tolerant species exposed to anoxic conditions had a greater increase in ADH activity, growth rate, energy charge and protein synthesis in leaves than in roots. They hypothesized that the shoot had a higher metabolic rate than the root in order to more rapidly grow to the surface of the overlying water. In contrast, less-tolerant species (eg. pea and maize) show an increased metabolic rate in the roots relative to leaves. In wild rice, ADH activity is greater in roots than in shoots after 2 d induction (more than twice as much) suggesting that this hypothesis is not true in this flood-tolerant species. Since ethanol was measured on a whole plant basis in our experiments the individual contribution of specific organs to ethanol synthesis cannot be determined from our data.

Three ADH isozymes are prominent in wild rice seed, whereas maize and barley seed express only a single isozyme of ADH (Freeling 1975, Good and Crosby 1989a). The expression of both ADH subunits in seeds may be a response to the need for higher ADH activity for hypoxic germination in wild rice. ADH activity in barley seed (7.0 IU/gm, Good and Crosby 1989a) and wild rice seed (9.4 IU/gm) is similar on a fresh weight basis, but considering wild rice seed must have at least 30% moisture content to remain highly viable (Elliot 1980), it would have substantially greater ADH activity (approximately two-fold higher) than barley seed on a dry weight basis.

The induction of LDH activity has been shown to occur in roots and seeds of other species exposed to hypoxic conditions (Smith and Ap Rees 1979, Hoffman et al. 1986, Good and Crosby 1989b) and is thought to have a transient role in lactate production (Davies 1980, Roberts et al. 1984). The fact that LDH is present at very low levels in ungerminated wild rice seed suggests that LDH does not have an important role in wild rice seed germination under hypoxic conditions, although LDH may be induced upon initiation of germination. LDH is inducible in root tissue but differs from ADH and AlaAT in being uninducible in leaf tissue (Fig. 5.1). The induction of LDH activity in roots occurs in the absence of any increase in lactate concentration in hypoxic seedlings. However, a very early transient increase in lactate concentration that might have occurred upon exposure of wild rice seedlings to hypoxic conditions may not have been detected at the first time point,1 d after imposition of hypoxia. Menegus et al. (1989, 1991) reported that flood-tolerant species such as rice and barnyard grass, do not show a dramatic drop in pH during flooding but instead undergo an alkalinization of the cytoplasm. Wild rice may also exhibit this characteristic, as the lack of appreciable lactate synthesis may be indicative of a cytoplasm that does not decrease in pH.

Under hypoxic conditions AlaAT activity is inducible in the roots of several cereals, but there is no activity increase in the leaf tissue in these species (Good and Crosby 1989b). In wild rice, AlaAT activity increases in both root and leaf tissue (Fig. 5.1). This increase is matched by a corresponding increase in alanine concentrations in both roots and leaves. Alanine concentrations are about two times greater in leaf tissue than in roots throughout the induction period, indicating that alanine may be important in maintaining hypoxic shoot growth in wild rice. Virtually every plant species studied to date, including *Oryza*, shows an increase in alanine pools in response to oxygen limited conditions (Effer and Ranson 1967, Streeter and Thompson 1972, Smith and ApRees 1979, Menegus et al. 1988). As described in Chapter 4, the role of alanine accumulation is thought to be involved in hypoxic nitrogen assimilation important for protein synthesis during and following hypoxic stress (Menegus et al. 1993, Vanlerberghe 1990). Menegus et al. (1993) suggest that the hypoxic accumulation of alanine in *Oryza* coleoptiles is important in the generation of osmotic pressure for the elongation of the coleoptile. This phenomenon may also be important in wild rice leaf extension, as leaf tissue accumulated more alanine than root tissue.

As discussed in Chapter 4, there is evidence that aspartate concentration decreases rapidly upon initiation of anaerobiosis and that there is a 1:1 molar relationship between the production of alanine and the depletion of aspartate early under conditions of anaerobiosis. In wild rice, however, aspartate levels are very low under hypoxic conditions, and the increase in alanine levels in both leaf and root tissue cannot be accounted for by decreases in the aspartate concentration.

Vanlerberghe et al. (1990) proposed that oxaloacetate pools produced by the transamination reactions described above are important in the net accumulation of succinate as seen in *S. minutum*. They state that succinate may accumulate through a modified tricarboxylic acid cycle from fumarate via oxaloacetate and malate, thus regenerating NAD^+ and producing a net gain of 2 moles ATP per mole of succinate produced. In this scheme malate would not accumulate but would be rapidly converted into fumarate. We showed that during hypoxia in wild rice malate concentration increased, whereas succinate concentrations were very low and did not increase indicating that the proposed modified tricarboxylic acid (TCA) cycle is not operating under hypoxic conditions in wild rice. Under hypoxic conditions malate levels generally decrease in other plants (Smith and ap Rees 1979, Menegus et al 1988). However, the relationship of high levels of malate accumulation coupled with insignificant levels of succinate and lactate in wild rice follows a pattern similar to that found in several flood-tolerant species studied by Crawford and Tyler (1969). They showed that malate concentrations increased, while lactate and succinate did not accumulate in 5 flood-tolerant species that were studied. The reason for malate accumulation in these plants is not apparent since, for example, ATP would not be generated by malate synthesis.

When the wild rice metabilite data is compared directly to that of barley in Chapter 4, many differences are evident. The obvious difference is that of the quantitative accumulation of ethanol in these two species. Barley accumulates approximately 160 μmol ethanol/gm of tissue over a 4-day period (Fig. 4.2), whereas wild-rice accumulates one-tenth that amount over the same period. In addition, the accumulation of alanine in barley roots is quite rapid, peaking after 24 h of hypoxic stress (Fig. 4.3). Wild rice accumulates alanine at a slower, linear rate in both leaves and roots (Fig. 5.3), and never reaches the concentrations found in barley roots.

There may be a couple of explanations for the differences in ethanol and alanine production in wild rice and barley. First, there may exist an hypoxic pathway in wild rice, not detected here, that produces ATP other than by ethanolic fermentation. Animal cells tolerant to hypoxia accumulate several end-products such as octopine, alanopine and propionate in order to increase the amount of ATP produced (Fields 1983). None of these compounds have been measured in hypoxic plant tissue and may be important in the hypoxic metabolism of wild rice. Alternatively, many tissues of the wild rice plant might dramatically decrease their metabolic rate, and channel their energy reserves into the growth of a particular tissue. *Oryza* coleoptiles are the only organs that significantly grow under strict anaerobic conditions. Although there is no obvious sign of coleoptile extension in hypoxically grown wild rice plants, perhaps leaf extension is accelerated.

A more likely explanation for the differences in ethanol and alanine production in wild rice and barley may be due to the differences in hydroponic growth conditions of these two species. Barley roots were submerged in nutrient media throughout the experiment with the leaves exposed to air. Wild rice, on the other hand, was completely submerged throughout the experiment in order to simulate natural growth. To allow photosynthesis to continue, the wild rice plants were grown in clear fish tanks, and to prevent algal growth in the wild rice tanks, nutrient solution was not provided. The wild rice plants were, therefore, dependent on their seeds for mineral nutrition. Since these plants were grown without an external mineral source for 10 d, consumption of seed reserves prior to the initiation of the experiment was perhaps high enough to decrease the metabolic rate. In comparable experiments by other workers, plants were often only 4 to 7 d old (e.g. Alpi and Beevers 1983, Menegus et al. 1988, Smith and ap Rees 1979). Since our seed supply was short, 10 d growth was required to obtain sufficient plant material. If indeed the availability of nutrients in wild rice was limiting, it did not seem to hinder the synthesis of malate or the hypoxic induction of ADH and AlaAT activity as these increased substantially throughout the experiment.

Despite these possible qualifications, it appears that ethanol synthesis is the most important product of hypoxic metabolism in wild rice. Alanine and malate also accumulate in significant amounts throughout the induction period. These compounds are probably significant in maintaining the viability of wild rice under long-term periods of hypoxic stress.

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6 General Discussion

Prior to the initiation of this research in 1990, little was known about plant alanine aminotransferases or the significance of alanine production in response to flooding. Hypoxic alanine biosynthesis was apparently universal among plants, suggesting that alanine might be important for cell viability. The induction of AlaAT also suggested a critical role for alanine in hypoxic plant survival. This thesis was undertaken in an attempt to elucidate the expression pattern and role of AlaAT as a component of the "anaerobic response". The experiments discussed in this thesis have contributed to the understanding of hypoxic AlaAT expression and alanine synthesis. While these experiments were being done, several other researchers made important discoveries. AlaAT has been purified and characterized from *Chlamydomonas* and *Panicum miliaceum* (Lain-Guelbenzer et al. 1991, Son et al. 1991), and recently it was hypothesized that the primary role of hypoxic alanine production is in the assimilation of nitrogen (Vanlerberghe et al. 1991, Menegus et al. 1993).

6.1 Hypoxic expression of AlaAT

Chapters 2 and 3 describe the identification and purification of the hypoxically inducible AlaAT-2 from barley roots and the expression pattern of this enzyme. AlaAT-2 is a homodimer composed of 50 kD subunits and is very specific in the reactions that it catalyzes. The hypoxic increase in AlaAT activity matches that of the AlaAT-2 protein. Like AlaAT-2 protein, AlaAT mRNA concentrations also increase during the initial period of hypoxic stress. The decrease in message levels after the first day of hypoxic stress, however, occurs while AlaAT protein continues to accumulate. This suggests either that the protein is very stable during hypoxic stress and/or the translation rate increases during the latter stages of hypoxic stress. The rapid increase in mRNA followed by a decline during the later stages of hypoxia is observed in some, but not all ANP mRNAs (Peshke and Sachs 1993, Russell and Sachs 1989). Rowland and Strommer (1986) showed that hypoxic increase in *Adh1* mRNA was due to an increase in transcription rate and to an increase in mRNA. Whether the accumulation of barley AlaAT-2 transcript is a result of an increase in transcription and transcript stability cannot be determined from our data. The presence of a conserved anaerobic response element in the upstream region of several ANP genes suggests that transcriptional activation is important in the regulation of these genes (Olive et al. 1991). Chapter 3 also discusses the isolation of a maize AlaAT gene that likely encodes the hypoxically inducible isoform of AlaAT. It will be interesting to see if this gene is associated with regulatory elements similar to those of other maize ANP genes.

6.2 Hypoxic accumulation of alanine and ethanol

The hypoxic accumulation of alanine during long-term hypoxic stress was shown in Chapters 4 and 5. Of all the metabolites assayed, only alanine and ethanol accumulated in both barley and wild rice over the long-term induction experiment. Studies with *Adh1*$^{-}$ maize plants best demonstrate the requirement of ethanol synthesis for hypoxic survival (Harberd and Edwards 1982), and the coincident accumulation of alanine suggests an important role of alanine in a plant's survival in hypoxic conditions.

6.3 The role of alanine in a general theory of flood tolerance

When a plant experiences flooded conditions, it responds by increasing its glycolytic rate (Faiz-ur-Rahman et al. 1974). Ethanol is the major metabolic product of hypoxic glycolysis (Chapters 4 and 5). The majority of ethanol produced diffuses from the plant tissue, thus eliminating the potential toxicity of this product. Other compounds such as lactate and succinate also result in a net synthesis of ATP when they are produced, but these compounds account for only a minor fraction of the carbon flow (Smith and ap Rees 1979, Menegus et al. 1993). As acidic end-products, these compounds may be toxic to the cell. Therefore, it appears that ethanolic fermentation in plants is the major contributor of hypoxic ATP production.

In addition to ethanol, plants also synthesize large amounts of alanine in response to flooding (Chapters 4 and 5). Vanlerberghe et al. (1991) and Menegus et al. (1993) showed that the production of alanine was important in the hypoxic assimilation of nitrogen in the alga, *Selanastrum minutum*, and in *Oryza*. In *S. minutum*, 93% of the nitrogen assimilated under anaerobic conditions is sequestered into alanine. Nitrogen assimilation in plants requires energy in the form of ATP, and under aerated conditions, sufficient ATP is present for the uptake of nitrogen into amino acids other than alanine. Despite the short supply of ATP under hypoxic conditions, it appears that plants have developed a strategy for nitrogen assimilation through the synthesis of alanine. Alanine produced by a plant under hypoxic conditions obtains its carbon skeleton from glycolytically synthesized pyruvate (Fig. 1.5). The generation of pyruvate by glycolysis produces the ATP and NADH required for the assimilation of NH_4^+, which is also needed for alanine synthesis. Therefore, hypoxic alanine production allows for low level anaerobic NH_4^+ assimilation while maintaining energy and redox balance. By assimilating nitrogen at no net cost, the plant has a reserve of nitrogen that it can draw upon when synthesizing proteins. As a result, the energy required for the minimal cellular processes that occur under hypoxic conditions, such as ANP synthesis and maintainance of membrane integrity, is provided primarily by alcoholic fermentation. The source of nitrogen is alanine. Alanine pools may also be important as a nitrogen source for the plant upon return to aerobic conditions.

This model of anaerobic survival fits well with the accumulation of metabolic end-products described in this thesis and in other studies. However, it does not explain how flood-tolerant plants survive longer than intolerant ones, as the latter group also produce large amounts of ethanol and alanine. Studies that extend beyond the scope of this thesis may provide the answer.

Prior to hypoxic cell death, the pH of plant cells drops dramatically (Roberts et al. 1984, Saint-ges et al. 1991). This acidification is probably due to the production of acidic end-products of energy metabolism, such as lactate. Menegus et al. (1989, 1991) demonstrated that the cytoplasm of flood-tolerant species is maintained at a higher pH after the imposition of hypoxic stress than intolerant species which showed a rapid drop in cytoplasmic pH. They proposed that this drop is due to several factors: the production of acidic compounds, leakage of compounds from the acidic vacuole, and the lack of synthesis of proton consuming compounds. They proposed that tolerant species are successful because they depend primarily on alcoholic fermentation, while flood-intolerant plants produce lactate in addition to ethanol.

It is known that, under hypoxia, exogenous glucose supplied to excised tissues increases the viability of the cells (Vartepetian et al. 1976). This indicates that carbohydrate availability may be an important factor in the survival of plant cells. In many flood-intolerant species, carbohydrate translocation to hypoxic tissue is limited by a reduction or inactivation of phloem function caused by the hypoxia. Rice coleoptiles have the unusual ability to transport carbohydrate from the endosperm under hypoxic conditions allowing fermentation to continue in that tissue (Atwell and Greenway 1987). Many rhizomatous species, such as the aquatic bulrush plant, contain a substantial amount of carbohydrate reserve in their rhizomes, and have the ability to survive hypoxic conditions up to several months (Settler et al. 1987). These species are thought to be successful in oxygen-free environments because they have sufficient substrate to allow for continued fermentation.

Resistance to cell acidification and carbohydrate availability may have important roles in flood-tolerant species, however, there may be several other factors important for flood tolerance. Different species may have evolved several cellular mechanisms to survive low oxygen conditions. Some plants may depend on one or two of these mechanisms whereas others may possess several of these traits, each contributing a small amount to the enhancement of hypoxic survival. It is therefore probable that flood tolerance is controlled by many genes, each having major or minor effects.

6.4 Future work on AlaAT and flood tolerance in plants

Our laboratory is continuing research on the expression and role of the hypoxically inducible AlaAT in flood tolerance. The isolation of an AlaAT genomic DNA clone described in Chapter 3 permits further analysis on the regulation of this gene. This clone contains at least 2 kb of sequence upstream of the transcription start site and probably contains the elements necessary for hypoxic activation of transcription. The sequence analysis of this gene will be completed followed by a functional analysis of the upstream regulatory sequences. Deletion of DNA sequence 5' to the transcription start site of the AlaAT gene will performed, and these deletions will be analyzed for their ability to regulate transcription in transient expression assays. In addition, footprint and gel mobility shift assays will also be performed in an effort to define elements important in the binding of transcription factors.

Most of the experiments attempting to identify factors that are important in conferring flood-tolerance have involved the identification of hypoxically produced metabolites or ANPs of different flood-tolerant species. Making conclusions from these types of experiments is difficult, since the genetic background of these plants are so different. Another approach would be to isolate flood-intolerant mutants of a plant species which has a moderate or high tolerance to flooding. These mutants would be compared to the wild-type plant for the production of specific metabolites and the synthesis of different enzymes. In addition the mutant gene(s) could be cloned in an attempt to identify their function. The only known mutants which affect flooding tolerance are the *Adh1*$^-$ and ATN mutants of maize discussed in Section 1.5.2.

Although a mutational analysis of the anaerobic response may be very useful in the isolation of many important genes, it may not be as effective in isolating AlaAT mutants. A basal level of AlaAT activity is important in the constitutive synthesis of alanine, therefore, null AlaAT mutants would likely be lethal. An alternative approach would be to genetically transform flood-tolerant plants with AlaAT gene constructs which can conditionally express AlaAT under hypoxic conditions. This would allow us to determine the importance of alanine synthesis under hypoxic conditions, without affecting aerobic growth. This type of experiment has been performed in an attempt to determine the effect of over-expression of LDH on the flood-tolerance of carrot (Andrew Hanson, personal communication). A 20-fold overexpression of LDH did not contribute to increased or decreased tolerance to hypoxia in this plant. However, this study differed from that proposed here, as the LDH gene was constitutively expressed from the cauliflower mosaic virus 35S promoter. Since we now have an AlaAT cDNA, we can use it to modify the expression of AlaAT under hypoxic conditions. We are generating AlaAT constructs which will be transformed into plants in order to increase or decrease expression of AlaAT. These constructs will contain the AlaAT cDNA sequence, in the sense or antisense orientation, under the regulation of an *Adh1* transcriptional control region. Since the *Adh1* control region is tightly regulated, being "off" under aerobic conditions and "on" when the plant is exposed to hypoxic conditions, these constructs will conditionally regulate AlaAT expression. These experiments will look at the effects of over-expression (sense constructs) and perhaps under-expression (antisense constructs) of AlaAT on the tolerance of plants to hypoxia.

6.5 Concluding remarks

The results of the experiments presented in this thesis suggest that:

1. Hypoxic AlaAT expression is regulated in a similar fashion to other ANPs.
2. Alanine biosynthesis may be an important component of the long-term hypoxic response in barley and wild rice.
3. The significance of alanine accumulation in conferring tolerance to hypoxia is not yet known.

4. The isolation of an AlaAT cDNA will allow a study of the role of alanine biosynthesis in a plant's ability to withstand hypoxic stress.
5. The isolation of an AlaAT genomic clone will allow us to continue to study the coordinate regulation of AlaAT with other anaerobically induced genes.

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7 Appendices

7.1 Hoagland nutrient solutions for hydroponic culture[*]

Salt	per liter stock solution[‡]
$Ca(NO_3)_2 \cdot 4\,H_2O$ [$CaCl_2$][†]	18.8g [11.76g]
$MgSO_4 \cdot 7\,H_2O$	10g
KNO_3 [KCl]	13.2g [8.95g]
$NH_4H_2PO_4$ [NaH_2PO_4]	2.4g [3.12g]
Fe EDTA Na_2 EDTA (33.3 g/L) NaOH (4 g/L) $FeCl_2 \cdot 4$ H2O (17.8 g/L) HCl (0.15 g/L)	20 mL
Micronutrients H_3BO_3 (2.86 g/L) $MnCl_2 \cdot 4\,H_2O$ (1.81 g/L) $ZnSO_4 \cdot 7$ H2O (0.22 g/L) Na_2MoO_3 (0.18 g/L) $CuSO_4 \cdot 5$ H2O (0.08 g/L)	2 mL

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[*] The pH of the solution was adjusted to 6.7 by the addition of NaOH.

[‡] Half-strength Hoagland solution used in the hydroponic system was made of 500 mL of stock solution in 20 L of water.

[†] Square brackets indicate salts substituted to prepare nitrate-free Hoagland solution.

7.2 Components of buffers used for the assay of metabolites. Standard curves were produced for each of the assays by measuring the absorbance change at 340 nm after known amounts of each metabolite were added to the assay buffer. Absorbance changes are due to the oxidation or reduction of NADH or NAD^+, respectively. All enzymes were purchased from Sigma Co.

Alanine:

100 μL 1 M Tris-HCl (pH 7.5)
20 μL 0.5 M 2-oxoglutarate
1μL 1 mM NADH
5 U LDH
50 μL sample
5 U AlaAT
H_20 to 1 mL

Change of absorbance is measured over 10 min.

Aspartate:

100 μL 1 M Tris-HCl (pH 8.0)
25 μL 0.5 M 2-oxoglutarate
1 μL 1 mM NADH
2.5 U aspartate aminotransferase
15 U malate dehydrogenase
100 μL sample
H_2O to 1 mL

Change of absorbance is measured over 30 min.

Ethanol:

200 μL of 0.5 M Tris-HCl (pH 9.0)
20 μL of 50 mM NAD^+
1 U aldehyde dehydrogenase
50 μL sample
H_2O to 1 mL

Change of absorbance is measured over 10 min.

Lactate:

100 μL 1 M Tris-HCl (pH 8.0)
20 μL 50 mM NAD$^+$
1.3 U AlaAT
50 μL 1 M Glutamate
5 U LDH
100 μL of sample
H_2O to 1 mL

Change of absorbance is measured over 10 min.

Malate:

50 μL 1 M Tris-HCl (pH 9.0)
20 μl 50 mM NAD$^+$
15 U malate dehydrogenase
5 U aspartate aminotransferase
20 μL 0.5 M glutamate
100 μL sample
H_20 to 1 mL

Change of absorbance is measured over 10 min.

Pyruvate:

100 μL 0.1 M Tris-HCl (pH 7.5)
1 μL 1 mM NADH
5 U Lactate dehydrogenase
50-200 μL sample
H_2O to 1 mL

Change of absorbance is measured over 20 min.

Succinate:

100 μL 0.1 M Tris-HCl (pH 8.0)
20 μL 0.5 M $MgSO_4$
1 μL 1 mM NADH
100 μL solution A
5 μL solution B
100 μL sample
H_2O to 1 mL

Solution A: 10 mg CoA, 15 mg ITP, 10 mg PEP in 5 mL H_20.

Solution B: succinyl CoA synthetase (50 U) in 1 ml of buffer (3.2 M ammonium sulphate/ 50 mM Tris-HCl, pH 7.5).

Change of absorbance is measured over 10 min.